IN DEFENSE OF QUAKING ASPEN (*POPULUS TREMULOIDES*): THE DISTRIBUTION AND ROLES OF PHENOLIC GLYCOSIDES AND EXTRAFLORAL NECTARIES WITHIN AND AMONG TREES

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IN DEFENSE OF QUAKING ASPEN (*POPULUS TREMULOIDES*): THE DISTRIBUTION AND ROLES OF PHENOLIC GLYCOSIDES AND EXTRAFLORAL NECTARIES WITHIN AND AMONG TREES.

A

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Abstract

I studied the concentrations of phenolic glycosides (PGs) from leaves with and without extrafloral nectaries (EFNs) in *Populus tremuloides* during an outbreak of the aspen leaf miner, *Phyllocnistis populiella*, in interior Alaska. *P. populiella* feeds on the contents of epidermal cells from both top (adaxial) and bottom (abaxial) surfaces of *P. tremuloides* leaves. The objective of this study was to assess the association of chemical and biotic defenses in *P. tremuloides* and their interaction with the insect herbivore *P. populiella*. The concentration of PGs (salicortin and tremulacin) was approximately 70% greater in leaves bearing EFNs than in those without EFNs from short trees (<2.5 m); leaves with and without EFNs did not differ significantly in PG concentration for tall trees (5-8 m). Leaf mining caused the induction of the foliar PGs following eight days of mining. There was no difference in the ability of leaves with and without EFNs to induce PGs in response to mining. The extent of mining damage was significantly and negatively related to the PG concentration, whereas EFNs were not related to the extent of mining. At the site level, I found no evidence for a tradeoff between these two putative forms of defense in *P. tremuloides.*

Table of Contents

List of Figures

List of Tables

General introduction

Populus tremuloides

Quaking aspen (*Populus tremuloides*) has the largest distribution of any tree in North America (Preston, 1976). It can be found from the Atlantic to the Pacific and Alaska to Mexico (Mitton and Grant, 1996). In the interior of Alaska it is primarily found on south facing hillsides and along ridgelines. *P. tremuloides* is a primary successional species that can quickly colonize an area that has experienced fire and other forms of disruption (Marr, 1961; Mitton and Grant, 1980). *P. tremuloides* serves a significant ecological role in that it provides habitat for numerous species and can act as a firebreak in the relatively contiguous boreal spruce forest in interior Alaska (Mitton and Grant, 1996; Engle, 1991).

P. tremuloides is a dioecious species, meaning that it has both male and female individuals. The sex of the individual tree can be determined by investigating the catkins which are formed in early spring, prior to full leaf emergence. *P. tremuloides* can also reproduce asexually. Lateral roots from one individual can send up many shoots, producing a new stand of trees that is actually a single individual or clone composed of many ramets.

Insect herbivores on *P. tremuloides*

P. tremuloides is prone to large insect outbreaks because the plants grow in vast, monodominant, largely even-aged, stands (Mattson et al., 1991). In interior Alaska, several different species of insect herbivores feed upon *P. tremuloides.* Some of the gall forming insects that affect *P. tremuloides* are mites, flies, and aphids (Holsten et al., 2001). The large aspen tortrix (*Choristoneura conflictana*) is also found in interior Alaska, and its larvae skeletonize both the upper and lower leaf surfaces. Other insects that typically affect *P. tremuloides* in interior Alaska include, leaf rollers (*Epinotia solandriana*), leaf beetles (*Cyrysomela spp.*), blotch miner (*Phyllonorycter ontario*) and the aspen leaf miner (*Phyllocnistis populiella*, Lepidoptera: *Gracillariidae*) (Holsten et al., 2001).

At the time this study was conducted, the aspen leaf miner (*Phyllocnistis populiella*, Lepidoptera: *Gracillariidae*) was undergoing an outbreak in the interior of Alaska. Starting in 1996, high densities of *P. populiella* on *P. tremuloides* were observed near Fairbanks (R. Werner, unpublished data), and by 2005 the area affected had expanded to exceed 260,000 ha (U.S. Forest Service, 2006). *P. populiella* overwinters as an adult, and, in interior Alaska, the adult leaf miners typically emerge in mid May to early June. Oviposition commences when aspen buds break and the first leaves are exposed. The eggs are laid singly on both the upper and lower surfaces of young, rapidly expanding leaves. The eggs then sink into the leaf tissue, and about a week after oviposition the larvae hatch directly into the epidermal tissue. Unlike most species of leaf miners, *P. populiella* feeds on the cells of the epidermis, mining the top (adaxial) and the bottom (abaxial) independently (Hering, 1951; Condrashoff, 1964) (Fig. i). The larvae remain on the side on which they hatched, and mining continues for 10-20 days

(Condrashoff, 1964). When the larvae form a pupal fold, which is generally located at the leaf edge, feeding has ceased. Wagner et al. (2008) determined that epidermal mining damage by *P. populiella* negatively affects growth of *P. tremuloides* and showed that different physiological effects occur when leaves are mined on the top compared to the bottom leaf surface. Leaves with mining damage on the bottom surface exhibited a significant reduction in their ability to photosynthesize, which was due to the failure of the stomata to open normally (Wagner et al., 2008).

Secondary metabolites as a means of defense for *P. tremuloides*.

Secondary metabolites, termed "secondary" because they do not appear to have a direct role in primary metabolism (Whittaker and Feeny, 1971), are commonly found in plants and other organisms which lack an adaptive immune system (Williams et al., 1989). The biological activity of these metabolites in plants suggests that they may serve as a form of defense against pathogens and herbivores (Fraenkel, 1959). The Optimal Defense Hypothesis (ODH) predicts that the within-plant allocation of secondary metabolites will be distributed to maximize fitness (Rhoades, 1979).

The major class of secondary metabolites in *P. tremuloides* is phenolics. This class includes condensed tannins and phenolic glycosides (PGs), which appear to play a role in *P. tremuloides* defense against insect herbivory (Lindroth and Hwang, 1996; Lindroth, 2001). The PGs salicortin and tremulacin have previously been shown to negatively impact the survival, development, growth, and feeding of a variety of Lepidoptera species (Bryant et al., 1987; Hemming and Lindroth, 1995; Hwang and Lindroth, 1997, 1998; Osier et al., 2000).

Secondary metabolites in *P. tremuloides* tend to vary at multiple spatial and temporal scales, and have both a genetic and an environmental basis (Hemming and Lindroth, 1999). Previous investigations into PG concentrations in *P. tremuloides* have focused on variation among clones (Lindroth and Hwang, 1996), trees from different age classes (Donaldson et al. 2006a) and among leaves at different developmental stages (Lindroth et al., 1987; Bingaman and Hart, 1993; Kleiner et al., 2003).

Plants can respond to herbivore attack with the induction of both direct and indirect chemical defenses (Karban and Baldwin, 1997). Previous research has demonstrated that foliar damage by insect herbivores rapidly induces secondary metabolites in plants (Clausen et al., 1989; Lindroth and Kinney, 1998; Osier and Lindroth, 2001). All of these studies, however, focused on foliar damage caused by insects that removed large portions of the leaf tissue or artificial defoliation. Prior to this study, the induction of phenolics as a result of leaf mining has not been investigated.

Extrafloral nectaries (EFNs)

In addition to chemical defenses, plants can incorporate biotic defense strategies such as the production of extrafloral nectaries (EFNs). EFNs are sugar-secreting glands that are found outside of the flower. They commonly occur on the rachis, petiole, stem, or in proximity to the reproductive parts of the plant (Bentley, 1977). EFNs can be found in more than 90 plant families worldwide (Koptur, 1992). EFNs are commonly visited by predators and parasitoids of plant herbivores, notably ants and wasps, which often results in a defensive mutualism (Bentley, 1977; Pemberton, 1992; Rudgers, 2004).

The expression of EFNs in *P. tremuloides* is highly variable. In this species, EFNs are typically expressed on only a subset of leaves and when expressed are generally located at the junction of the petiole and the leaf (Trelease, 1881). The number of EFNs on the leaf can vary from 0 to 6, with most leaves possessing 0 or 2 (Wagner and Doak, unpublished data). The expression of EFNs also varies with leaf age (Doak et al., 2007). On preformed leaves, the first five to eight leaves in *P. tremuloides* that appear in spring, EFNs typically occur on the most proximal leaf, but decline in frequency on the more distal leaves. On neoformed leaves, those formed during the current year's growth, the EFN frequency is typically high (Doak et al., 2007).

EFNs on *P. tremuloides* may provide for a defense against the insect herbivore *P. populiella.* Doak et al. (2007) observed that leaves bearing EFNs experienced lower levels of mining damage than leaves without; however, this result was dependent upon tree size, with only short ramets (1-2m) receiving the benefit. EFNs on *P. tremuloides* may mediate protection mutualisms with ants, parasitoid wasps and predatory mites, (Mortensen, Wagner and Doak, unpub.).

However, the reduction in leaf mining observed on leaves bearing EFNs by Doak et al. (2007) may be due, in part, to an association between EFNs and chemical defense compounds such as the PGs.

Defense traits within and among *P. tremuloides*

Defenses within aspen trees

The fact that plant defense characters are not evenly distributed throughout a plant is widely hypothesized to be a strategy for cost savings (Feeny, 1976; Coley et al., 1985; Mondor et al., 2006). The Optimal Defense Hypothesis (ODH) predicts that leaves which are more valuable (i.e., having a greater impact on plant fitness) should be more heavily defended (McKey 1974, 1979; Rhoades, 1979). If a leaf were to employ two different defenses, this may result in a higher level of defense than if just one is employed (Berenbaum et al., 1991; Stapley, 1998). While previous studies have investigated EFN nectar secretion and PG concentrations and determined that these traits generally follow ODH predictions (Lindroth et al., 1987; Bingaman and Hart, 1993; Kleiner et al., 2003; Koptur, 1992; Heil et al., 2000; Wäckers et al., 2001; Mondor and Addicott, 2003), no one has investigated the association between the presence or absence of EFNs and PGs.

Defenses among aspen trees

The allocation of defenses among trees tends to differ from what is observed within trees. When a plant possesses more than one form of defense, and these traits are costly, negative correlations among these traits can be expected (Feeny, 1976; Coley et al., 1985). In previous studies, both phenolic glycosides and EFNs have been shown to be costly (Lindroth and Hwang, 1996; Mondor et al., 2006); therefore a negative correlation between these traits may be expected (Zangerl and Bazzaz, 1992; Mole, 1994).

Objectives of the study

The primary objective of this thesis is to assess the association of chemical and biotic defenses in *P. tremuloides* and their interaction with the insect herbivore *P. populiella*. The field work was carried out at 12 different aspen stands located in and around Fairbanks, Alaska. The goals of my thesis were to:

a) Evaluate the association between PGs and extrafloral nectaries within *Populus tremuloides* both within and among ramets.

b) Determine the effect of leaf mining by *P. populiella* on the foliar phenolic glycoside concentrations of P*. tremuloides.*

c) Determine how leaf mining damage by *P. populiella is* related to the concentrations of foliar PGs and the presence or absence of EFNs.

Fig. i. Damage caused by the aspen leaf miner.

Chapter 1: The association between phenolic glycosides and extrafloral nectaries within and among individuals of *Populus tremuloides*[1]

1.1 Abstract:

We studied the foliar concentration of phenolic glycosides (PGs) from leaves with and without extrafloral nectaries (EFNs) in *Populus tremuloides* Michx., across multiple sites during an outbreak of the aspen leaf miner, *Phyllocnistis populiella*, in the boreal forest of interior Alaska. *P. populiella* feeds on the contents of epidermal cells from both the top (adaxial) and bottom (abaxial) surfaces of *P. tremuloides* leaves. We observed that the average foliar PG concentration was five times higher in short (<2.5 m) ramets than in tall (>2.5 m) ramets. Within short ramets, the concentration of PGs in EFN-bearing leaves was approximately 70% greater than in leaves without EFNs. This was not the case, however, for tall ramets, which had similar levels of PGs in EFN and non-EFN bearing leaves. The foliar concentration of PGs had a significant negative relationship with the percentage of leaf surface damaged by *P. populiella*, although PGs explained only 2-3% of the variation in leaf mining. In

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[1] Young, B. D., Wagner, D., Doak, P., Mortenson, B., and T. P. Clausen (2009). The association between phenolic glycosides and extrafloral nectaries within and among individuals of *Populus tremuloides.* Prepared for submission *to Oecologia.*

contrast, the presence of EFNs was not significantly related to a reduction in leaf surface mining for either the top or bottom leaf surfaces. Among sites the mean concentration of foliar PGs and the mean EFN frequency were not correlated; therefore, we found no evidence for a tradeoff between these two putative forms of defense in P. *tremuloides.*

1.2 Introduction:

The interactions between plants and herbivores have led to the evolution of a wide variety of plant defenses. Typically, an individual plant possesses more than one way of defending itself (Duffey and Stout, 1996). Defenses which affect susceptibility to, or severity of, attack (e.g., thorns, tricomes and plant secondary compounds) are generally referred to as 'direct'. Defenses that can attract, house, or feed natural enemies of plant herbivores (e.g., extrafloral nectaries and volatile organic compounds; review Heil, 2008) are commonly referred to as 'indirect'. Extrafloral nectaries (EFNs), for example, are sugar producing glands that have been shown to mediate defensive mutualisms between plants and predacious arthropods, including ants and parasitoid wasps (Bentley, 1977; Bronstein, 1998; Koptur, 1992, 2005). Direct and indirect defense traits can operate independently or work in concert with other traits (Agrawal and Fishbein, 2006).

The forest tree species *Populus tremuloides* produces both indirect and direct defenses. Extrafloral nectaries (EFNs) appear on a subset of leaves and are located at the junction of the petiole and the leaf blade (Trelease, 1881). Most leaves possess either 0 or 2 EFNs (Doak et al., 2007). The frequency of EFN expression on *P. tremuloides* leaves in the interior of Alaska varies along shoots, among trees within a site, and among sites (Doak et al., 2007). The first five to eight leaves that emerge from the bud in spring, called the preformed leaves, vary considerably with respect to presence and absence of EFNs. The two most proximal leaves on a shoot, which unfurl a day or two before the more distal leaves, typically possess at least one EFN. The frequency of EFN occurrence subsequently declines on the more distal preformed leaves (Doak et al., 2007). In contrast, the leaves which are initiated and expand during the growing season, called the neoformed leaves, typically possess EFNs (Doak et al., 2007). Preformed leaves that possess EFNs suffer lower levels of leaf mining herbivory than leaves with no EFNs (Doak et al., 2007).

P. tremuloides leaves also produce phenolic glycosides (PGs), which can act as a form of direct defense. PGs are effective at reducing the performance of, and damage caused by, several important herbivore species of *P. tremuloides* (review Lindroth, 2001). Concentrations of PGs vary among leaves within *P. tremuloides* plants; for example, the concentration of PGs within leaves tends to be higher in younger leaves than in older leaves (Lindroth et al., 1987; Bingaman and Hart, 1993; Kleiner et al., 2003).

When two different forms of defense exist, as is the case for *P. tremuloides,* how should they be distributed among leaves within a tree? The

Optimal Defense Hypothesis (ODH) predicts that tissues that have a greater impact on individual fitness are more heavily defended (McKey, 1974, 1979; Rhoades, 1979). The few studies that have investigated the relative distribution of multiple defenses have supported ODH predictions, finding high levels of both defenses on leaves predicted to be of high value to the plant. Traw and Feeny (2008) reported that two direct defenses of mustard, trichomes and glucosinolates, were concentrated on young leaves and on those that made the greatest contribution to light capture relative to other leaves. In addition, Radhika et al. (2008) observed that two indirect traits, volatile organic compounds and EFN nectar secretion, were both highest in leaves that contributed more towards future photosynthesis.

Predictions of the ODH may also apply to the distribution of PGs and EFNs among the leaves of *P. tremuloides.* The most proximal leaves on a shoot, which tend to have EFNs, unfurl first in the spring and therefore may be especially important to carbon capture and expansion of the more distal leaves. In addition, EFNs themselves may contribute to the value of a leaf. Experimental evidence from our research group indicates that EFNs attract crawling arthropods that reduce herbivory, and that leaves with EFNs contribute to the defense of surrounding leaves lacking EFNs (B. Mortensen, unpublished data). In addition, EFNs are permanent structures that may be costly to produce (Mondor et al., 2006); hence it may be profitable to defend EFN-bearing leaves more heavily through chemical means. If the ODH applies to *P. tremuloides,* we would expect to find high PG concentrations in EFN-bearing leaves relative to leaves lacking EFNs.

However, high concentrations of secondary compounds might interfere with a leaf's ability to attract predacious arthropods to EFNs. Many studies have documented ecological costs of plant chemical defense (review Strauss et al., 2002). These studies have chiefly focused on the cost of repelling pollinators; however, it is possible that such compounds could also repel predators from EFN-bearing leaves. If such an incompatibility between EFNs and PGs occurs in *P. tremuloides,* we would expect to find lower PG concentrations on leaves bearing EFNs relative to those lacking EFNs.

The relationship between EFNs and PGs may change as plants grow. Doak et al. (2007) demonstrated that smaller *P. tremuloides* ramets had higher EFN frequencies than tall ramets. In addition, leaves with EFNs sustained lower levels of herbivory than leaves without EFNs on short ramets, but not on tall ramets, suggesting that the benefit of EFNs declines as plants grow. Numerous studies have demonstrated that younger *Populus* ramets also have higher concentrations of PGs than older ramets (Lindroth et al., 1987; Bingaman and Hart, 1993; Kleiner et al., 2003; Donaldson et al., 2006). Smaller *P. tremuloides* ramets tend to have less foliage than that of taller ramets; therefore each leaf may be more valuable to the plant and more highly defended (Dirzo, 1984). If the distribution of PGs is influenced by the value of EFN-bearing leaves, then a positive relationship between the two might be expected to break down in large trees.

A secondary goal of this study is to better understand the relative distribution of EFNs and PGs among plants. In contrast to the within-plant predictions, current hypotheses on the evolution of plant defenses predict negative correlations between defensive traits among plants when these traits are costly (McKey, 1988; Zangerl and Bazzaz, 1992; Mole, 1994). For example, Rehr et al. (1973) found that Acacia trees protected by ants have much lower levels of cyanogenic glycosides than non-ant acacias (but see Heil et al., 2002). Additionally, Eck et al. (2001) found these same patterns with condensed tannins in the genus *Macaranga.* In aspen, tree growth is strongly negatively correlated with the combined concentrations of foliar PGs and tannins (Lindroth and Hwang, 1996), suggesting that PGs are costly to produce. There is no such direct evidence for a cost of EFN production for any EFN bearing plant species, though indirect evidence suggests that such costs exist (Rutter and Rausher, 2004, Mondor et. al., 2006). If indeed both PGs and EFNs are costly to produce, and the defenses work in an additive rather than synergistic manner, a negative correlation between these traits might be expected among *P. tremuloides* genets.

During the period of our study, an outbreak of the aspen leaf miner (*Phyllocnistis populiella*, Lepidoptera: Gracilliadae) was occurring in interior Alaska. Starting in 1996, high densities of *P. populiella* on *P. tremuloides* were observed locally (R. Werner, unpublished data), and by 2005 the area affected had expanded to exceed 260,000 ha (U.S. Forest Service, 2006). Unlike most species of leaf miners, *P. populiella* feeds on the cells of the epidermis, from both the top (adaxial) and the bottom (abaxial) leaf surfaces independently (Hering, 1951; Condrashoff, 1964). The developing larvae is unable to tunnel downward or exit and reenter the leaf due to prognathus mouthparts; thus it feeds on a single side of the leaf throughout its development (Hering, 1951). Wagner et al. (2008) determined that epidermal leaf mining damage negatively affects growth of *P. tremuloides* and showed that different physiological effects occur when leaves are mined on the top compared to the bottom leaf surface. When leaves were mined on only the bottom leaf surface, there was significant reduction in the leaves' ability to photosynthesize, which was due to the failure of the stomata to open normally, while top mining appeared to have no effect on photosynthesis (Wagner et al., 2008). Mining damage to either the top or bottom leaf surface induces the production of the phenolic glycosides salicortin and tremulacin in the foliar tissue (B. D. Young, unpublished data).

Here we examine natural patterns of EFN occurrence and phenolic glycoside concentration within trees from multiple sites to address four questions: (i) How are PGs distributed among leaves with and without EFNs? (ii) Does the relationship between PGs and EFNs differ with ramet height? (iii) Are the presence of EFNs and the concentration of foliar PGs associated with lower levels of epidermal leaf mining by the aspen leaf miner? And (iv) is there a negative relationship between the frequency of leaves bearing EFNs and average foliar PG concentrations across sites?

1.3 Materials and Methods:

1.3.1 Field Methods

We quantified the occurrence of EFNs, the concentration of PGs, and the amount of natural mining damage on ramets within twelve *P. tremuloides* stands (Table 1.1) located in and around Fairbanks, Alaska (64°48’N, 147°42’W) during June of 2007. Within each site, we haphazardly chose a set of six short ramets (0.5 – 2.5 m in height). At four sites (WR, CR, ED and RF) we selected an additional set of four tall (5.0 – 8.0 m height) ramets. Ramets at each site were located within a 10 m x 10 m area and appeared to belong to a single clone based on spatial separation from other putative clones, growth form, and leaf morphology; however, we were not able to conclusively determine that ramets at each site comprised a single genotype.

At each site, we sampled leaves 11 days following the onset of leaf mining. The sites were sampled on different days because of the variation in leaf miner phenology among sites (Table 1.1). The leaves were sampled on day 11 because this is when the majority of leaves (>90%) had at least one leaf miner settling into a leaf fold in preparation for pupation, indicating that some of the leaf miners had ceased to feed. Previous work indicated that PGs induced by *P. populiella* mining damage was evident by day 11, so damaged leaves sampled in this study likely possessed induced levels of PGs (B. D. Young, unpublished data). We sampled three leaves from each of three haphazardly selected shoots on each ramet. Leaves were collected from the 2nd, 3rd, and 4th leaf positions from the proximal end of the shoot, which typically possess the greatest variability in EFN expression (Doak et al., 2007). Leaf samples were cleanly snipped from the petiole, immediately placed into 10 ml of 50% (aqueous) methanol solution, and stored on ice for transport to the lab. We maintained the leaf samples in solution at 2°C ± 1°C for 24 hours. The leaves were then removed from the extract solution and placed into a plant press. The extract was stored at -40°C until analysis.

For each leaf we recorded the number of EFNs and visually estimated the percent leaf mining on both surfaces of the leaf and the percent of leaf tissue missing and or damaged by other forms of herbivory to the nearest one percent. Our visual estimates of mining correlate well with measurements made with image analysis software ($R^2 > 0.9$, Doak et al., 2007). Leaves were scanned using a desk top scanner, and leaf area was measured using image analysis (Scion Image, Fredrick, Maryland, USA). Leaves were then dried at 60°C for one week and weighed to the nearest 0.1 mg.

To better estimate the frequency of EFNs at each site we examined leaves from an additional 15-18 short (0.5 - 2.5 m in height) ramets at each of the 12 sites. These ramets were adjacent to the ramets that were sampled for chemical analysis. Leaf samples were collected from leaf positions 1 through 6 from the proximal end of the shoot for each of three different shoots per ramet. For each leaf, we recorded the presence and number of EFNs. The site level EFN frequencies were then calculated by averaging the number of leaves with EFNs across the total number of leaves sampled at each site.

1.3.2 Chemical Analysis

We prepared the aspen leaf extracts for analysis by filtering a 1.0 ml aliquot of extract through a 0.45 µm pore acrodisc. The samples were injected onto a 4.6 x 250 mm XDB-C8 column (Agilent) attached to a High Performance Liquid Chromatography (HPLC) (Agilent 1100) equipped with a UV/VIS diode array detector (Agilent) and analyzed at 230 nm. The PGs were separated using a mobile phase gradient of acetonitrile (CH_3CN) and H_20 with a constant flow rate of 1.0 ml/min. The gradient elution was: 1% CH_3CN (0–4 min), 1– 60% CH_3CN (4–10 min), 60 – 80% CH_3CN (10–15 min), 100% CH_3CN (15–22 min) and 100 – 1% CH_3CN (22–27 min). This was followed by a 10 min period at 1% CH_3CN prior to the injection of next sample. The PGs, salicortin and tremulacin, were quantified using purified reference standards.

1.3.3 Data Analysis

To investigate the distribution of chemical defense compounds among leaves with and without EFNs within trees, we applied a mixed model analysis of covariance (ANCOVA) with PG concentration (the sum of salicortin and tremulacin concentrations) as the dependent variable, EFNs (dichotomous: presence vs. absence) as a fixed effect, and date sampled and leaf position as covariates. Site, the interaction of site and EFNs (site x EFNs), ramet, and shoot were included as random effects. To determine if the relationship between PGs and EFNs differed in large and small ramets, we used an additional ANCOVA with EFNs (dichotomous), tree height (dichotomous: short vs. tall), and EFNs x tree height as fixed effects, and date sampled and leaf position as covariates. Site, site x EFNs, ramet, and shoot were again included as random effects.

To investigate the association between leaf mining damage and both PG concentration and the presence and absence of EFNs, we used mixed model ANCOVAs with EFNs (dichotomous); PGs and their interaction terms as fixed effects; sampling date and leaf position as covariates; and site, site x EFNs, site x PGs, ramet, and shoot as random effects. In addition to the ANCOVAs, we evaluated the relationship between leaf mining and PG concentrations on leaves with and without EFNs using correlations. Separate analyses were performed for top and bottom mining.

To explore the site level relationships between the mean site level PG concentrations, percentage of leaf surface mined at each site, and the site level EFN frequencies, correlations were calculated. Separate calculations were performed for top and bottom mining.

We used scatter diagrams of residuals and normal probability plots to verify parametric assumptions. Dependent variables were log transformed when necessary to meet parametric assumptions. The mixed model analyses were conducted using PROC MIXED (SAS Institute Inc., Cary, North Carolina) and the correlations were conducted using JMP IN version 5.1.2 (SAS Institute, Cary, North Carolina, USA)

1.4 Results

1.4.1 Distribution of PGs and EFNs among leaves within ramets

For short ramets, leaves bearing EFNs contained significantly higher concentrations of PGs than leaves without EFNs (Fig 1.1; $F_{1,9}$ =11.67; P< 0.01). Foliar PG concentrations did not vary significantly with leaf position (positions 2-4) along the shoot ($F_{1,350}$ =1.29; P= 0.25). Although we attempted to collect leaves from different sites when they were at similar phenological stages, the concentration of the foliar PGs was significantly and positively related with sampling date (Fig. 1.1; $F_{1,350}$ = 15.05, P< 0.001). However, there was considerable variability, with some late sites having relatively low concentrations of PGs.

Using the smaller set of four sites at which leaves were collected from both short and tall trees, we found that both the foliar PG concentrations and the distribution of PGs relative to EFNs varied with tree height. The average foliar PG concentration of short ramets (<2 m) was nearly an order of magnitude higher than that of tall (5-8 m) ramets (Fig. 1.2; $F_{1,210}$ =86.74; P < 0.001). Overall, EFNs did not explain a significant amount of the variation in PGs in this data set ($F_{1,3}$ =

2.05; P=0.24); however, there was a significant interaction between ramet height and EFNs ($F_{1, 210}$ = 9.78; P<0.01). In tall ramets, leaves with and without EFNs did not differ significantly in PG concentration (Fig 1.2; Tukey–Kramer *P* > 0.05), but in short ramets, the EFN-bearing leaves had higher concentrations of PGs than leaves lacking EFNs (Fig 1.2; Tukey–Kramer *P* = 0.02). Foliar PG concentrations did not vary significantly with leaf position ($F_{1, 350}$ =1.29; P= 0.25). In this data set, there was no significant difference in the PG concentration across sampling days, most likely because the sites were sampled on three consecutive days ($F_{1, 210}$ = 0.97; P=0.32).

1.4.2 Patterns of leaf mining damage

PG concentration explained a significant portion of the variation in leaf mining damage. On both the top and bottom leaf surfaces, the mining damage to the leaf was significantly and negatively related to the total foliar PG concentration (Fig 1.3: Bottom leaf surface: $F_{1, 9}$ = 16.08, P < 0.01; EFN leaves: r= -0.24, df = 328, P< 0.01; No EFN leaves: r= -0.27, df = 212, P< 0.01; Top leaf surface: $F_{1, 9}$ = 14.07, P < 0.01; EFN leaves: r= -0.22, df = 328, P< 0.01; No EFN leaves: r= -0.08, df = 212, P= 0.20). Although this relationship was statistically significant, PG concentration explained only a small portion (2-3%) of the variation in leaf mining among leaves. Examination of Fig 1.3 suggests that there may have been a threshold in PG concentration, above which PGs were more effective at deterring mining. However, a piecewise-regression model did not result in an improved r^2 value.

Mining damage was not significantly related to the presence of EFNs (Bottom: $F_{1,9} = 0.03$; P= 0.87; Top: $F_{1,9} = 0.45$; P= 0.51), nor was there a significant interaction between EFNs and PGs (Bottom: $F_{1,339} = 0.42$; P= 0.51; Top: $F_{1,339} = 0.67$; P= 0.41). Mining damage did not vary systematically across leaf positions (Bottom: $F_{1,339} = 0.11$, P= 0.74; Top: $F_{1,339} = 0.89$, P= 0.34) or sampling dates (Bottom: $F_{1,339} = 0.05$; P= 0.82; Top: $F_{1,339} = 0.51$, P= 0.47).

1.4.3 Site level patterns of EFNs, PGs, and epidermal leaf mining

Among the sites, there were no significant correlations between EFNs, PGs and epidermal leaf mining. The average EFN frequencies, which were calculated from a different set of short (<2.5 m in height) ramets at the 12 different sites than was used for the chemical analysis, were not correlated with the mean site PG concentration (r = 0.29; df = 10; P > 0.05). Because foliar PG concentration changed over the sampling period, the variation due to sampling date could potentially interfere with our ability to detect a relationship between PGs and EFN frequency. Therefore, we also tested whether the residuals from the regression of PGs versus sampling date correlated with EFN frequency, and again found no significant association between the two (r = 0.11; df = 10; P > 0.05). There was no significant correlation between the average PG concentration and the average leaf mining damage for either the top or bottom leaf surfaces among sites (Leaf top: r = -0.13; df = 10; P > 0.05 and leaf bottom r = - 0.21; df = 10; P > 0.05), nor was the mean site EFN frequency significantly correlated with epidermal leaf mining (Top: r = -0.02; df = 10; P > 0.05 and Bottom r = -0.25; df = 10; P > 0.05).

1.5 Discussion

Within short ramets (<2.5 m in height) of *P. tremuloides* in interior Alaska, we found that within site and sampling date leaves bearing EFNs contained a significantly greater average concentration of PGs than leaves without EFNs. While previous studies have shown variation in chemical compounds within individual plants as a result of shoot length (Suomela, 1996), foliar development (Osier et al., 2000), foliar vascular architecture (Orians and Jones, 2001), location in the canopy (Fortin and Mauffette, 2002), and leaf size (Roslin et al., 2006), this is the first study, to our knowledge, that demonstrates a positive correlation between leaves bearing EFNs and foliar PGs within individual plants.

The results are similar to those of previous studies investigating the distribution of dual defenses among leaves on a plant (Traw and Feeny, 2008; Radhika et al., 2008), and appear to support the predictions of the ODH. The higher concentrations of PGs in EFN-bearing leaves may reflect the value of this subset of leaves to the ramet as a whole, either because of their early expansion or because the EFNs themselves warrant further chemical defense. In a related study, the protection provided by EFNs on short ramets was shown to extend to leaves lacking EFNs (Mortensen unpubl. data). In addition, EFNs may be costly to produce (Rutter and Rausher, 2004; Mondor et al., 2006). The higher concentrations of PGs found in leaves bearing EFNs may therefore be an attempt by the tree to further defend these more valuable leaves. Alternatively, EFNs and PGs may function in a synergistic manner.

The relationship between EFNs and PGs observed in the small ramets did not occur in tall ramets (>2.5 m in height). This difference may be related to the effectiveness of EFNs on short and tall trees. Natural levels of leaf mining are lower on the EFN-bearing leaves of short ramets relative to leaves without EFNs (Doak et al. 2007), and experimental evidence indicates that a higher rate of leaf miner predation and parasitism is responsible for a portion of this difference (Mortensen, unpubl. data). Moreover, a leaf with EFNs on a short tree provides a measure of protection to surrounding leaves lacking EFNs (Mortensen, unpubl. data). In contrast, EFNs are not associated with lower herbivory on tall trees (Doak et al., 2007). Because EFNs appear to benefit short ramets more than large ones, on short ramets the profitability of investment in chemical defense may be greater for EFN-bearing leaves than for leaves without EFNs. However, we note that because we sampled fewer tall ramets than short ones, we also had less power to detect differences between leaves with and without EFNs on tall ramets.

At a mechanistic level, there may be a functional connection between nectar production and phenolics glycoside production. PGs are thought to be synthesized from the pool of available sugars within the apoplastic space (Arnold and Shultz, 2002). EFNs are supplied by vascular tissue composed of both xylem and phloem (Elias et al., 1975; De la Barrera and Nobel, 2004), and subsequently a build up of carbohydrates can occur within the leaf parenchyma before the carbohydrates can be broken down and secreted as nectar (Elias et al., 1975). This possible additional pool of carbohydrates may be used by the leaf for the further production of the PGs (Arnold et al., 2004).

The five-fold lower foliar concentration of PGs on tall relative to short ramets may help to explain why Doak et al. (2007) observed higher levels of epidermal mining by the aspen leaf miner on leaves from tall ramets. This pattern provides further support for the predictions of the ODH (McKey 1974, 1979; Rhoades, 1979). Our findings are similar to those of Donaldson et al. (2006), who reported a negative relationship between foliar phenolic glycoside concentrations and ramet age from *P. tremuloides* clones growing in Wisconsin.

Numerous studies have investigated the role of PGs as a primary form of defense in *P. tremuloides* (Lindroth, 2001; Donaldson et al., 2006; Osier and Lindroth, 2006; Donaldson and Lindroth, 2007; Stevens et al., 2007). These studies have included specialist and generalist insects, both outbreak and non-outbreak species, and have been conducted in both laboratory and field settings. The PGs salicortin and tremulacin have been shown to negatively impact the survival, development, growth, and feeding of a variety of other Lepidoptera species (Bryant et al., 1987; Hemming and Lindroth, 1995; Hwang and Lindroth, 1997, 1998; Osier et al., 2000). In our study, PGs explained a significant portion of the variation in leaf mining damage caused by *P. populiella*.

Two potential mechanisms might give rise to the negative relationship between epidermal leaf mining damage and foliar PG concentration. First, PGs might act as an oviposition suppressant to *P. populiella*. PGs have been found to act as an oviposition suppressant for some herbivore taxa. For example, in a study by Roininen and Tahvanainen (1989), PGs reduced oviposition on willow by a specialist sawfly. In addition, within willow, oviposition rate by a leaf beetle was negatively related to the concentration of tremulacin (Bingaman and Hart, 1993). Bingaman and Hart (1992) also demonstrated a strong relationship between the amount of leaf damage and the number of beetle eggs laid on the leaf. In contrast, some specialized insect herbivores use PGs as a stimulus for oviposition (Orians et al., 1997; Roininen et al., 1999). The influence of PGs on the oviposition behavior of *P. populiella* is unknown.

Second, PGs may reduce the performance of *P. populiella* larvae. Previous studies relating the PGs salicortin and tremulacin to herbivore performance revealed that these compounds are an important form of resistance for *P. tremuloides* against numerous generalist and specialist foliar feeding Lepidoptera, including *Pachysphinx modesta*, *Choristoneura conflictana*, and *Malacosoma disstria* (reviewed in Lindroth, 2001). Kao et al. (2002) determined that phenolic compounds are present in the epidermal tissue of *P. tremuloides*; therefore it is possible that *P. populiella* are exposed to PGs while feeding.

Previous work on this study system showed leaf mining damage by *P. populiella* was lower on leaves with EFNs compared to those without (Doak et. al., 2007). Our results suggest that the lower damage to EFN-bearing leaves may result, in part, from higher average concentrations of PGs in these leaves. We found no evidence of lower herbivory due to the presence of EFNs in our survey of 12 sites. However, this result should be interpreted with caution due to collinearity between EFNs and PG concentrations, which could mask the full effect of EFNs (Scheiner and Gurevitch, 1993). In addition, because some epidermal mining continued at the study sites after the leaves used in this study were sampled, the total influence of EFNs on mining damage may not have been captured in our sampling.

At different hierarchical scales, the allocation patterns of chemical defense compounds within trees tend to vary (Suomela, 1996; Hwang and Lindroth, 1997; Osier et al., 2000; Orians and Jones, 2001; Fortin and Mauffette, 2002; Boege and Dirzo, 2004; Roslin et al., 2006). The lack of a negative relationship among sites suggests that there is not a tradeoff between direct (PGs) and indirect (EFNs) defense traits in our study population. Wooley et al. (2007) determined that *P. tremuloides* ramets from within a common garden composed of twelve genotypes also failed to exhibit a negative relationship between EFN densities and foliar PGs concentrations.

In conclusion, *P. tremuloides* in the interior of Alaska contains higher levels of PGs in leaves bearing EFNs than in leaves lacking EFNs within a site and by sampling date. The concentration of PGs is negatively, although weakly, related to the extent of epidermal leaf mining damage. While the function of EFNs in this system is still not clear, the relationship between leaves bearing EFNs and the level of PGs is notable. In a field study conducted by Doak et al. (2007), *P. tremuloides* leaves bearing EFNs had lower leaf mining damage on average than leaves lacking EFNs; both chemical and biotic defenses of EFN-bearing leaves may have contributed to this result. However, further investigation will be necessary to understand the effects of PGs and the role of EFNs on *P. populiella* development and performance.

1.6 Acknowledgments

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1.7 References

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Table 1.1 Characteristics of twelve naturally occurring *P. tremuloides* stands located in and around Fairbanks, Alaska. The percentage of bottom (% B. Min.) and top (% T. Min.) leaf mining and the foliar phenolic glycoside concentrations (mg/g leaf tissue) [PGs] are the mean (± 1 SE) values from six short ramets (0.5 – 2.0 m height) per site. The EFN frequencies (EFN freq.) are the mean (± 1 SE) values from 15 – 18 different short ramets (0.5 – 2.0 m height) per site.

Site	Date sampled	Latitude (N)	Longitude (W)	Elevation (m)	% B. Min.	% T. Min.	[PGs]	EFN freq.
BB	5/24/07	64°42’26”	148°18’18”	201.1	23 ± 3	9 ± 1	0.41 ± 0.05	0.42 ± 0.03
BR	5/26/07	64°42’30”	148°18’18”	204.8	38 ± 4	37 ± 4	1.64 ± 0.20	0.46 ± 0.03
BS	5/31/07	64°42’57”	148°19’58”	225.2	23 ± 5	27 ± 5	2.80 ± 0.29	0.35 ± 0.03
CH	5/30/07	64°53’53”	147°30’51”	222.2	16 ± 4	9 ± 2	0.32 ± 0.02	0.73 ± 0.03
CP	6/04/07	64°50’25”	147°52’82”	140.2	3 ± 1	3 ± 1	0.44 ± 0.02	0.52 ± 0.03
CR	6/05/07	64°55’08”	147°43’19”	383.1	8 ± 3	13 ± 3	2.97 ± 0.21	0.72 ± 0.03
DH	5/31/07	64°49’06”	147°58’40”	252.9	33 ± 4	41 ± 4	1.65 ± 0.18	0.82 ± 0.03
ED	6/06/07	64°52’60”	148°03’85”	719.3	41 ± 5	17 ± 2	5.06 ± 0.35	0.34 ± 0.03
PE	5/31/07	64°47’54”	148°09’06”	460.2	11 ± 3	11 ± 2	1.12 ± 0.16	0.51 ± 0.03
RF	6/04/07	64°53’59”	147°48’03”	322.7	46 ± 5	58 ± 5	4.37 ± 0.26	0.79 ± 0.02
RP	6/01/07	64°49’70”	147°57’86”	304.8	3 ± 2	4 ± 1	4.47 ± 0.25	0.63 ± 0.03
WR	6/05/07	64° 51’46”	147°51’27”	191.1	10 ± 3	4 ± 1	5.50 ± 0.26	0.75 ± 0.03

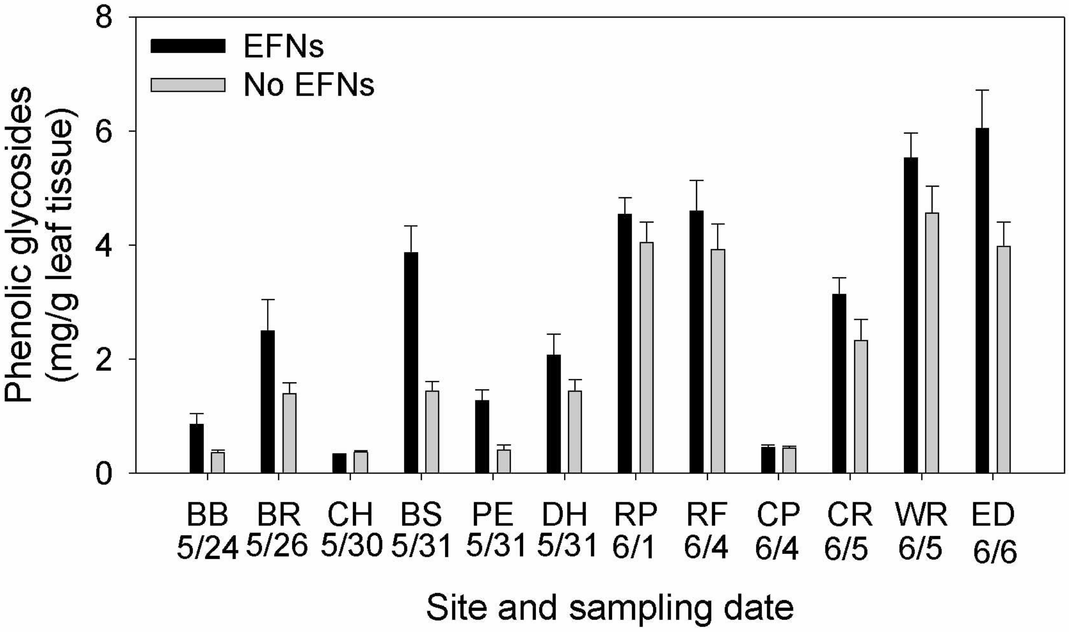

Fig. 1.1 Foliar concentrations of PGs for leaves with and without EFNs and their relationship to sampling date. Bars show the average PG concentration of short (< 2.5 m) ramets from twelve sites in and around Fairbanks, Alaska. Dark bars represent leaves with EFNs; light bars represent leaves without EFNs. Error bars indicate +1 SE.

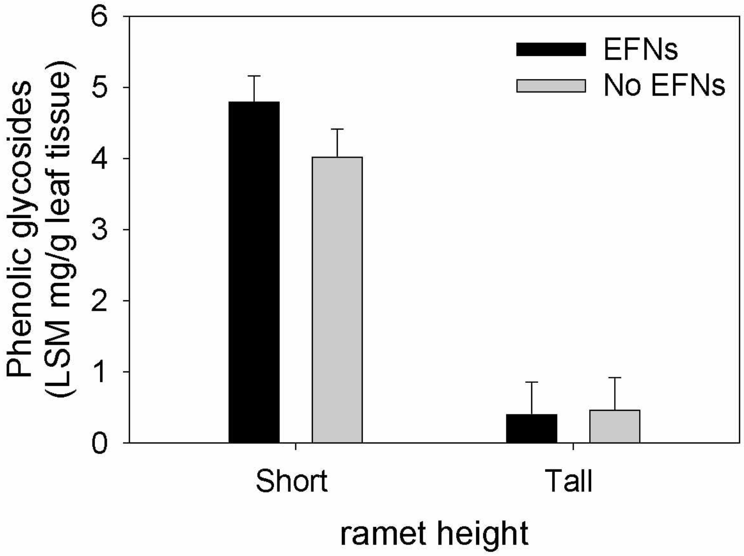

Fig. 1.2 Least square mean concentrations of PGs from short (< 2.5 m) and tall ramets (> 5.0 m) from four sites located within and around Fairbanks, Alaska. Dark bars represent leaves with EFNs; light bars represent leaves without EFNs. Error bars indicate +1 SE.

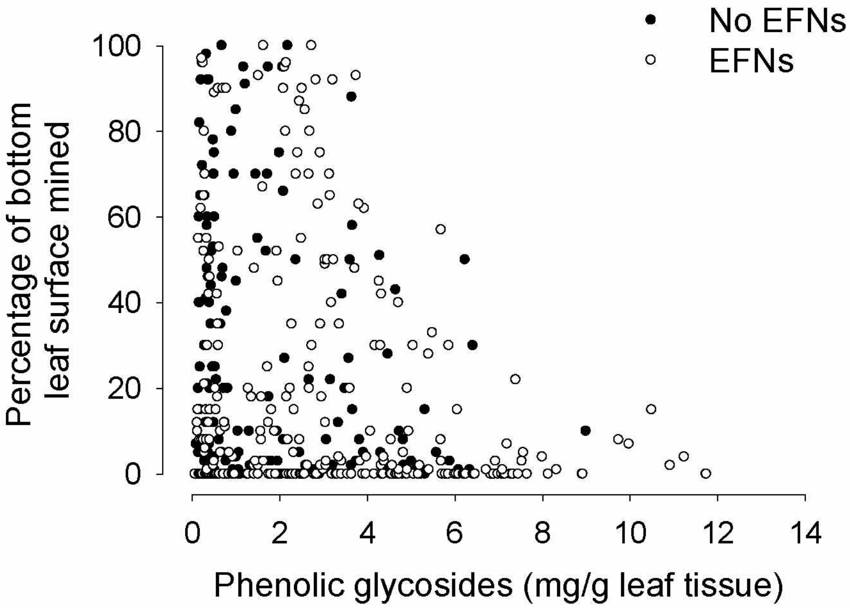

Fig. 1.3 Percentage of bottom leaf surface mining by *P. populiella* from short (< 2.5 m) *P. tremuloides* ramets with varying levels of foliar phenolic glycosides. Data points are from leaves with (open circles) and without (closed circles) EFNs from leaf positions 2-4 from the shoot. Pearson's correlation coefficient: EFN leaves r= -0.24, df = 328, P< 0.01; No EFN leaves: r= -0.27, df = 212, P< 0.01

Chapter 2: Epidermal leaf mining by *Phyllocnistis populiella* induces phenolic glycosides in quaking aspen[1]

2.1 Abstract:

We studied the effect of epidermal leaf mining on the leaf chemistry of quaking aspen, *Populus tremuloides* Michx., during an outbreak of the aspen leaf miner, *Phyllocnistis populiella* in the boreal forest of interior Alaska. *P. populiella* feeds on the epidermal cells of *P. tremuloides* leaves. Concentrations of the phenolic glycosides (PGs) tremulacin and salicortin were significantly higher in leaves that had received natural levels of leaf mining than in leaves sprayed with insecticide to reduce mining damage. The induction response was not observed until the eighth day following the onset of leaf mining and continued through day eleven, the last day of sampling. Leaves with extrafloral nectaries (EFNs) had significantly higher constitutive levels of PGs than leaves lacking EFNs, but there was no difference in the ability of leaves with and without EFNs to induce PGs in response to mining. The extent of mining damage to leaves was significantly and negatively related to the total foliar PGs concentration. While several species

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[1] Young, B. D., Wagner, D., Doak, P., and T. P. Clausen (2009). Epidermal leaf mining by *Phyllocnistis populiella* induces phenolic glycosides in quaking aspen. Prepared for submission to *Journal of Chemical Ecology*

from the leaf-chewing guild of insects have been shown to induce phenolic glycoside concentrations, this is the first such study to show induction of PGs in response to leaf mining.

2.2 Introduction:

Some plants respond to herbivore damage by inducing the production of chemical defenses, a response that can help to protect the remaining tissue against further damage (Karban and Baldwin, 1997). Induction of resistance traits may have evolved in response to the high cost of producing chemical defenses, allowing the plant to avoid investing energy and nutrients in defense unless it is subjected to damage (Rhoades, 1979). For plants that experience heavy competition, such as immature *Populus tremuloides* trees (Bokalo et al., 2007), such cost savings may allow a greater proportion of resources to be invested in early growth and survival.

Various phenolic compounds function as a form of chemical defense in a wide assortment of terrestrial plants. The concentration of these compounds can vary greatly among plant parts, individuals, populations, and species (Krischik and Denno, 1983) and tend to exhibit extensive temporal variation (Mattson and Scriber, 1987; Slansky, 1993; Osier et al., 2000). The concentrations of phenolic compounds in plant tissues, as well as their temporal development, are determined in part by genetic factors (Roth et. al., 1998; Osier et. al., 2000;

Lindroth et. al., 2002). In addition, environmental factors such as herbivory can also dramatically influence the levels of phenolic concentrations in plant tissue through the mechanism of induction (Karban and Baldwin, 1997; Agrawal et al., 1999). Induction of phenolics varies within and among plant species and can range from rapid and localized induction (Clausen et al., 1989; Zangerl and Berenbaum, 1995; Stout et al., 1996) to slow and systemic induction (Lindroth and Kinney, 1998; Osier and Lindroth, 2001; Stevens and Lindroth, 2005).

The primary chemical defense compounds in *Populus tremuloides* are the phenolic glycosides (PGs) salicortin and tremulacin (Lindroth, 2001). These compounds have been shown to be highly effective at reducing the performance of a number of generalist and specialist insect herbivores through negative effects on development, growth, feeding, survival, and reproduction. (e.g., Bryant et al., 1987; Lindroth and Hwang, 1996a, b; Ayres et al., 1997; Osier and Lindroth, 2001; Donaldson and Lindroth, 2007). PGs can exhibit extensive genetic variation among clones and temporal variation within clones, individuals, and different plant parts (Lindroth et al., 1987; Hemming and Lindroth, 1995; Lindroth and Hwang, 1996a, b; Osier and Lindroth, 2001; Donaldson and Lindroth, 2007). PGs also respond to resource availability (e.g., light, H_2O, CO_2) (Hemming and Lindroth, 1999). The induction of PGs in *P. tremuloides* as a result of herbivory has been reported in numerous studies which primarily focused on tissue removal through simulated defoliation or by insects from the leaf-chewing guild (e.g., Bryant et al., 1987; Clausen et al., 1989, 1991; Lindroth and Hemming, 1990; Hemming and Lindroth, 1995, 1999; Hwang and Lindroth, 1997, 1998; Osier et al., 2000). However induction by epidermal leaf mining insects has not been previously studied.

Some plant species (e.g., *Leonardoxa*, *Macaranga, Acacia,* and *Populus)* which produce phenolic defense compounds also possess extrafloral nectaries (EFNs) (Heil et al., 2002; Wooley et al., 2007). In contrast to chemical defenses, which can have direct effects on the performance of herbivores, EFNs can function as an indirect resistance trait by luring arthropod predators and parasites of herbivores onto the plant (Bentley, 1977; Koptur, 1992; Röse et al., 2006). In some populations, EFNs appear to increase plant fitness by reducing tissue loss to herbivores (Heil et al., 2004; Rudgers, 2004).

P. tremuloides, in addition to producing PGs, bears EFNs on a subset of its leaves (Doak et al., 2007). The proportion of leaves bearing EFNs can be increased as an induced response to herbivory; however, induction of EFNs is much slower than induction of PGs (Wooley et al., 2007). The presence of EFNs on aspen leaves of small aspen ramets is associated with lower levels of leaf mining in interior Alaska (Doak et al., 2007). EFN-bearing leaves of small aspen ramets have higher average concentrations of PGs relative to leaves lacking EFNs (B. D. Young, unpublished data), suggesting that direct, chemical defense may contribute to the pattern of reduced herbivory on EFN-bearing leaves.

From the late 1990s through 2008, *P. tremuloides* in Alaska and western Canada experienced a severe outbreak of the aspen leaf miner (*Phyllocnistis populiella*, Lepidoptera: *Gracillariidae*) (Fig. 2.1A)) (U.S. Forest Service, 2006). Unlike most leaf mining herbivores, *P. populiella* feeds only on the cells of the leaf epidermis, leaving the mesophyll tissue intact (Condrashoff, 1964) (Fig. 2.1B). *P. populiella* larvae mine the top and bottom surfaces of the leaf independently. Wagner et al. (2008) determined that mining reduces the growth of *P. tremuloides*. While mining of the top surface had few serious physiological effects, mining on the bottom surface, where the stomata are located, reduced photosynthesis (Wagner et al., 2008).

In this study, we investigated PG concentrations and EFN expression in aspen to address two specific questions: (*i*) does epidermal leaf mining by *P. populiella* induce the PGs salicortin and tremulacin, and (*ii*) do constitutive and/or induced concentrations of PGs differ in leaves with and without EFNs?

2.3 Materials and Methods

2.3.1 Natural History

P. tremuloides has the largest distribution of any tree in North America. It can be found from the Atlantic to the Pacific and Alaska to Mexico (Mitton and Grant, 1996). In interior Alaska it tends to be found on south facing hillsides and along ridgelines. Aspen can reproduce both sexually and asexually, resulting in the formation of clonal stands.

EFNs in *P. tremuloides* are located at the junction of the petiole and the leaf (Trelease, 1881) and are typically expressed on only a subset of its leaves. The number of EFNs on a leaf can vary from 0 to 6, with most leaves possessing 0 or 2 (Wagner and Doak, unpublished data). The proportion of leaves bearing EFNs varies within and among trees. Within trees, EFN expression varies with developmental leaf type. Preformed leaves are initiated the previous season and held dormant as embryonic leaves within the leaf bud (Critchfield, 1960); they are the first five to eight leaves to emerge in spring. In contrast, neoformed leaves are produced and expand during the growing season and, when they occur, extend the shoot beyond the preformed leaves. The frequency of occurrence of EFNs on neoformed leaves is higher and more consistent than on preformed leaves (Doak et al., 2007). Within preformed leaves, the most proximal leaves usually bear EFNs while the distal leaves do not. Among trees, EFN expression varies among stands and is more common on shorter (<2 m) trees than on taller trees (>4 m) (Doak et al., 2007).

Aspen leaves lacking EFNs sustain more damage from the leaf miner *Phyllocnistis populiella* (Lepidoptera: *Gracillariidae*) (Doak et al. 2007)*. P. populiella* overwinters as an adult, and in interior Alaska the adult leaf miners emerge in early to mid May. Oviposition occurs shortly after bud break on the host tree. The eggs are laid singly on both the upper and lower surfaces of young rapidly expanding leaves. The eggs then sink into the leaf tissue, and about a week after oviposition the larvae hatch directly into the epidermal tissue. The developing larvae feed on the cells of the leaf epidermis, leaving obvious tracks or mines. The larvae remain on the side on which they hatched, and mining continues for roughly10 days on both the top and bottom of the leaf surfaces independently. When the larva forms a pupal fold, which is generally located at the leaf margin, feeding ceases (Condrashoff, 1964).

2.3.2 Field Methods

To investigate the effects of epidermal leaf mining on aspen phenolic glycoside concentrations in leaves with and without EFNs, we first experimentally reduced leaf miner densities on a set of aspen ramets using an insecticide and then compared leaf salicortin and tremulacin concentrations to those of control ramets. The following year, we investigated how the concentrations of salicortin and tremulacin changed during the period of active feeding by the leaf miner.

In early June of 2006 we haphazardly chose a set of 12 small ramets (0.8 – 1.2 m in height) near the summit of Ester Dome (64°52’56”N, 148°03’57”W, elevation 720 m, hereafter ED) near Fairbanks, Alaska. Ramets were located within a 20 m x 20 m area and exhibited similar physical characteristics; however, we cannot be certain that they all belonged to a single clone. Of the 12 ramets, half were assigned at random to receive an insecticide treatment. Subsequent to oviposition, the treatment ramets were sprayed with the insecticide spinosad (Conserve; Dow AgroSciences, Indianapolis, Ind.; concentration 1.56 ml l^{-1}; applied with a hand-powered pump sprayer until runoff). The insecticide was then reapplied 6 days later. When the treatment ramets were sprayed with the insecticide, control ramets were sprayed with an equal quantity of water.

We sampled the leaves eleven days following the onset of leaf mining. At this point, more than 90% of the control leaves possessed one or more pupal folds, indicating that many of the aspen leaf miner larvae had ceased feeding and were preparing to pupate (Condrashoff, 1964). We sampled preformed leaves from the five most proximal leaf positions from three haphazardly chosen shoots per ramet. We harvested leaves by cleanly snipping them at the petioles. Leaf samples were immediately placed into 10 ml of 50% MEOH (aq) and stored on ice during transport to the lab. We maintained the leaf samples in solution at 2°C ±1°C for 24 hours. The leaves were then removed from the extract solution and pressed in a plant press. The extract was stored at -40°C.

For each leaf, we counted the number of EFNs and visually estimated leaf mining on the top and bottom surfaces of the leaf and the percent of leaf tissue missing and/or damaged to the nearest 1.0%. Our visual estimates of mining correlated strongly with measurements made using image analysis software (R^2

> 0.9, Doak et al., 2007). The total percentage of the leaf surface mined was calculated as the percent top plus bottom mining and divided by two. Leaves were scanned using a desk top scanner, and leaf area was measured using the image analysis program Scion Image (Fredrick, Maryland, USA). Leaves were dried at 60°C for one week and weighed to the nearest 0.1 mg.

To determine how foliar PGs concentrations changed on a daily basis during the period of feeding by *P. populiella*, we conducted a second experiment at the same study site in May 2007. We chose 42 ramets ranging in size from 0.5 to 1.6 m height, all located within a 25 m x 25 m area adjacent to the area used 2006. Again, the genetic identity of the plants was not known. We assigned half of the ramets at random to receive an insecticide treatment. Insecticide was applied to treatment ramets, and water to controls, in the same manner as the previous year. To accommodate repeated sampling while avoiding the removal of excessive numbers of leaves from individual ramets, we used a larger number of ramets than in 2006 and sampled each ramet on only two occasions. Leaf samples were collected on days 1, 2, 3, 4, 5, 8, and 11 after the onset of leaf mining was observed on any of the control leaves. On each sampling day, we sampled preformed leaves from leaf positions 2 and 3 on a single shoot from 6 control and 6 treatment ramets, chosen at random from the set of ramets that had not yet been sampled. Leaves were again sampled by cleanly snipping leaves at the petioles. Mattson and Palmer (1988) did not find a chemical response from the ramet when the leaf was sampled in this manner. When each ramet had been sampled once, we resampled each ramet a second time, using a different shoot. All leaf samples were handled and processed as in the previous year.

2.3.3 Chemical Analysis

We prepared the aspen leaf extracts for analysis by filtering a 1.0 ml aliquot of extract through a 0.45 µm pore acrodisc. The samples were injected onto a 4.6 x 250 mm XDB-C8 column (Agilent) attached to a High Performance Liquid Chromatography (HPLC) (Agilent 1100) equipped with a UV/VIS diode array detector (Agilent) and analyzed at 230 nm. The PGs were separated using a mobile phase gradient of acetonitrile (CH_3CN) and H_20 with a constant flow rate of 1.0 ml/min. The gradient elution was: 1% CH_3CN (0–4 min), 1– 60% CH_3CN (4–10 min), 60 – 80% CH_3CN (10–15 min), 100% CH_3CN (15–22 min) and 100 – 1% CH_3CN (22–27 min). This was followed by a 10 min period at 1% CH_3CN prior to the injection of next sample. The PGs, salicortin and tremulacin, were quantified using purified reference standards.

2.3.4 Data Analyses

We tested the effect of leaf mining on concentrations of the PGs salicortin and tremulacin at the end of 11 days of mining (2006 data) using separate mixed model analyses of covariance (ANCOVA) with insecticide treatment, EFNs (dichotomous: absence vs. presence) and their interaction as fixed effects, leaf position as a covariate, and shoot within ramet and ramet as random effects.

To assess changes in salicortin and tremulacin concentrations over time following the onset of mining, we used separate mixed model ANCOVAs with insecticide treatment as a fixed effect, day sampled as a categorical variable, leaf position as a covariate, and ramet as a random effect. Too few leaves lacking EFNs were sampled in 2006 to include EFNs in the statistical model. Within each sampling date, the foliar PG concentrations of the sprayed and control ramets were compared using planned contrasts.

In both data sets, we tested for non-target effects of the insecticide treatment by comparing the percent leaf damage caused by herbivores other than *P. populiella* using Wilcoxon tests. Dependent variables were log transformed when necessary to meet parametric assumptions. Mixed model analyses used the restricted maximum likelihood method and were conducted using JMP IN version 5.1.2 (SAS Institute, Cary, North Carolina, USA).

2.4 Results

The insecticide treatment prevented all mining damage by *P. populiella* on the sprayed ramets during both 2006 and 2007 (Table 2.1). Across all experimental trees, leaf mining represented the greatest source of leaf damage but chewing, skeletonizing, and galling damage were also observed. Leaf damage due to herbivores other than *P. populiella* was not significantly reduced by the insecticide treatment (Table 2.1).

At the end of 11 days of mining in 2006, leaves that sustained natural levels of leaf mining damage had significantly higher concentrations of both PGs tremulacin and salicortin than leaves from ramets sprayed with the insecticide (Fig. 2.2; tremulacin: $F_{1,137} = 11.37$, $P = < 0.001$; salicortin $F_{1,137} = 6.88$, $P < 0.001$). Across treatments and ramets, leaves with EFNs had significantly higher mean concentrations of tremulacin and salicortin than leaves lacking EFNs (Fig. 2.2; tremulacin: $F_{1,137} > 17.80$; $P < 0.001$; salicortin: $F_{1,137} = 8.03$; $P < 0.001$). The lack of significant interaction terms indicates that there was no difference in the ability of leaves with or without EFNs to induce PGs in response to mining (Fig. 2.2; tremulacin: interaction $F_{1,137} = 0.184$; $P = 0.66$; salicortin: interaction $F_{1,137} = 0.165$; $P = 0.68$). Foliar phenolic glycoside concentrations increased significantly along the shoot, with the most distal leaves having the highest concentrations (Fig. 2.3; tremulacin: $F_{1,137} = 7.57$; $P < 0.001$; salicortin: $F_{1,137} = 7.13$; $P < 0.001$).

During the 2007 growing season, we investigated the time course of induction in relation to mining. The concentration of salicortin in leaves varied significantly over the course of the eleven day sampling period (Fig. 2.4a; $F_{6,111} = 8.43$; $P = <0.001$). During the first five days, salicortin concentrations in both sprayed and control leaves rose, peaking at day three, and then fell (Fig. 2.4a). The level of salicortin in sprayed trees showed no significant increase after day five. Eight and eleven days following the onset of leaf mining activity, trees with natural mining levels had significantly higher concentrations of salicortin than leaves of sprayed trees (Fig. 2.4a; LS means contrast: $F = 7.69$; $P < 0.05$).

The foliar concentrations of tremulacin in 2007 also varied significantly over time (Fig 2.4b; $F_{6, 111} = 5.649$; $P < 0.001$). During the first three days there was minimal change in the foliar concentration of tremulacin. On day four, and continuing through day five, tremulacin concentration dropped in both sprayed and control leaves (Fig. 2.4b). As with salicortin, the average foliar concentration of tremulacin on naturally mined trees rose after day five and was significantly higher than in controls on days 8 and 11 (Fig. 2.4b.). The increase in tremulacin and salicortin on days eight and eleven corresponded to about 5% of the total leaf surface damaged by mining (Fig. 2.4c).

2.5 Discussion

Damage to the epidermis caused by the mining activity of *P. populiella* led to the induction of the PGs salicortin and tremulacin in *P. tremuloides* leaf tissue. Induction of PGs occurred while leaf miner larvae were still feeding, and therefore could potentially affect insect performance. Epidermal leaf miners are taxonomically restricted to a small subset of leaf miner taxa (Hering, 1951). The vast majority of leaf mining species feed primarily on the cells of leaf mesophyll (Hering, 1951), which has previously been shown to cause the induction of putative defensive proteins (Stout et al., 1994; Inbar et al., 1999). Studies investigating the induction of phenolic compounds as a result of mesophyll leaf mining, however, have produced negative results in other species (Fisher et al., 2000; Ramiro et al., 2006). The present study is the first, to our knowledge, to document the induction of phenolic compounds as a result of feeding by a leaf mining herbivore.

Previous studies have shown considerable variation in the timing of induction of phenolic compounds as a result of tissue damage within *P. tremuloides* (Haukioja, 1990; Clausen et al., 1991; Parry et al., 2003). In this study, the induction of salicortin and tremulacin was not detected until more than five days following the onset of damage (Fig. 2.4). A lag time between damage and PGs induction is not uncommon for *P. tremuloides*. For example, after imposing a combination of simulated and insect defoliation, Stevens and Lindroth (2005) reported that it took several months before an induced response was observed. It is possible that the apparent lag time for induction in our study could be a result of leaf temporal ontogeny. The very young aspen leaves may not have been photosynthetically competent. In this case, the leaves would have been dependent on their sink strength for incorporating additional phenolic glycoside precursors (Jones et al., 1993). This may have limited their ability to elicit an induced response (Arnold and Schultz, 2002).

Foliar concentrations of PGs within *P. tremuloides* are temporally variable (Osier et al., 2000; Lindroth et al. 2002). The relatively high concentration of PGs in our study for both the sprayed and control leaves (Fig. 2.4) during the first few days was likely due to the up-regulation of these compounds during leaf development. Osier et al. (2000) observed, within two separate *P. tremuloides* clones in Wisconsin, the highest foliar concentration of PGs in newly-emerged leaves. In addition, Julkunen-Tiitto et al. (1995) found that the concentrations of many PGs are highest in the young shoot tips of *S. myrsinifolia*. The reduction in the concentration of the PGs after day three from our study may reflect the dilution of PGs as the leaf gained mass (Jones and Hartley 1999). Alternatively, the initial rise in PG concentration in young leaves might reflect induction due to damage caused by newly-hatched leaf miner larvae prior to insecticide application. However, we find this possibility unlikely, because the subsequent decline in PG concentration after day three occurred in both sprayed and control trees.

The percentage of leaf surface damage observed in our study (Fig. 2.4c), is lower than has been previously observed for the induction of secondary metabolites (review Karban and Baldwin, 1997). However, these previous studies focused primarily on tissue loss caused by leaf chewing or mechanical defoliation. While aspen is prone to extensive defoliation during insect outbreaks (Mattson et al., 1991), herbivory rarely exceeds 10% on a year to year basis (Coupe and Cahill, 2003). The induction of the PGs at a low level of damage may provide an advantage for the plant in that it may drive away other herbivores or affect the performance of those that are currently feeding (Karban and Baldwin, 1997). Induction, however, can also carry with it a cost when the level of herbivory does not have a great enough impact on the plant to affect its overall fitness (Agrawal et al., 1999); therefore, a threshold of damage has been suggested (Underwood, 2000).

The constitutive and induced concentrations of tremulacin and salicortin were greater in leaves bearing EFNs compared to those without EFNs. Covariance in EFNs and PGs within and among ramets is addressed in Chapter 1. The high concentrations of phenolic glycosides in EFN-bearing leaves may contribute to the lower levels of damage by the leaf miner *P. populiella* reported by Doak et al. (2007). We found no difference in the ability of leaves with and without EFNs to induce PGs in response to epidermal mining.

In conclusion, our results demonstrate that epidermal mining by *P. populiella* induces the production of the PGs tremulacin and salicortin. While the role of PGs or EFNs in deterring herbivory was not investigated in this study, we note that an induced response does occur while the larvae are still feeding. Further investigation will be necessary to understand both the constitutive and induced effects of PGs and EFNs on *P. populiella* development and performance.

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Table 2.1 Percent leaf damage (mean ± SE) by *P. populiella* and other herbivore taxa after experimental reduction in leaf miner abundance 11 days post treatment. "Other damage" is the sum of chewing, skeletonizing, and galling. Mean values for each ramet were compared with Wilcoxon signed rank tests.

	2006			2007		
	Control	Sprayed	P	Control	Sprayed	P
Top mining	61 ± 3	0	***	18 ± 3	0	***
Bottom mining	44 ± 3	0	***	17 ± 4	0	***
Other damage	1 ± 1	1 ± 1	n.s.	1 ± 1	1 ± 1	n.s.

n.s. P> 0.05, *** P<0.001

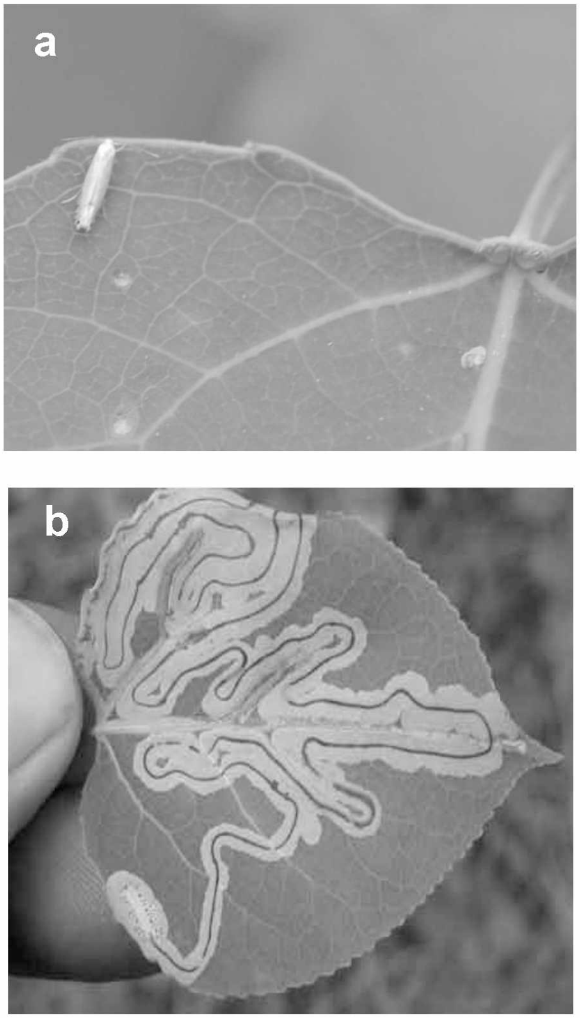

Fig. 2.1 (a) Aspen leaf miner moth and eggs on a leaf with two EFNs at the petiole. (b) Epidermal leaf mining by the aspen leaf miner on a leaf without EFNs.

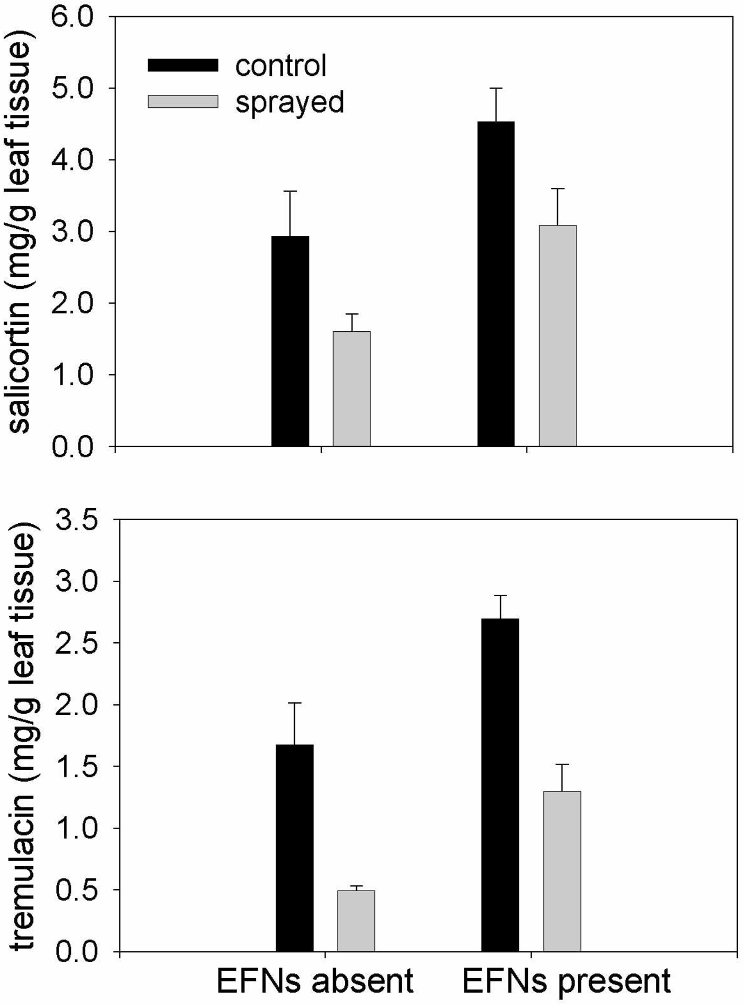

Fig. 2.2 Mean (±SE) concentrations of salicortin and tremulacin from naturally mined (control) and insecticide-sprayed leaves with and without EFNs during the summer of 2006.

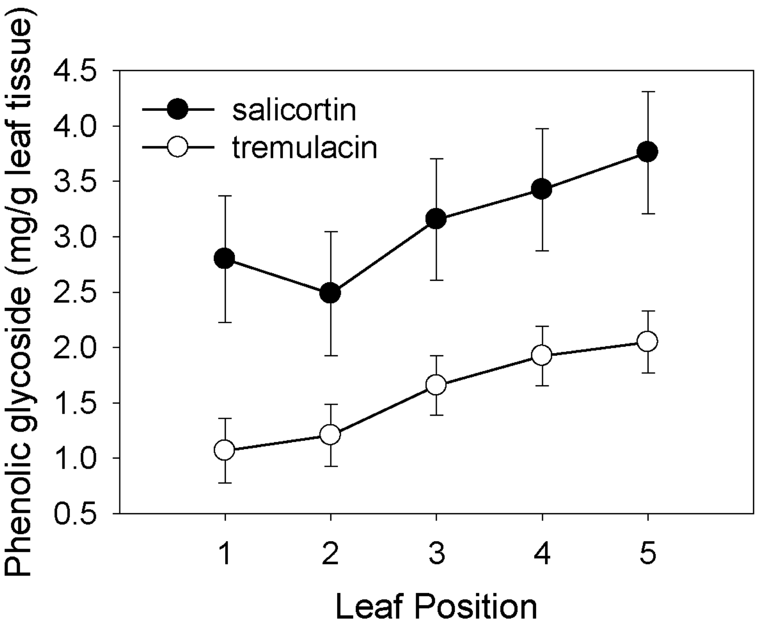

Fig. 2.3 Least square mean (±SE) concentrations of salicortin and tremulacin from naturally mined (control) preformed leaves from leaf positions 1 through 5 during the summer of 2006

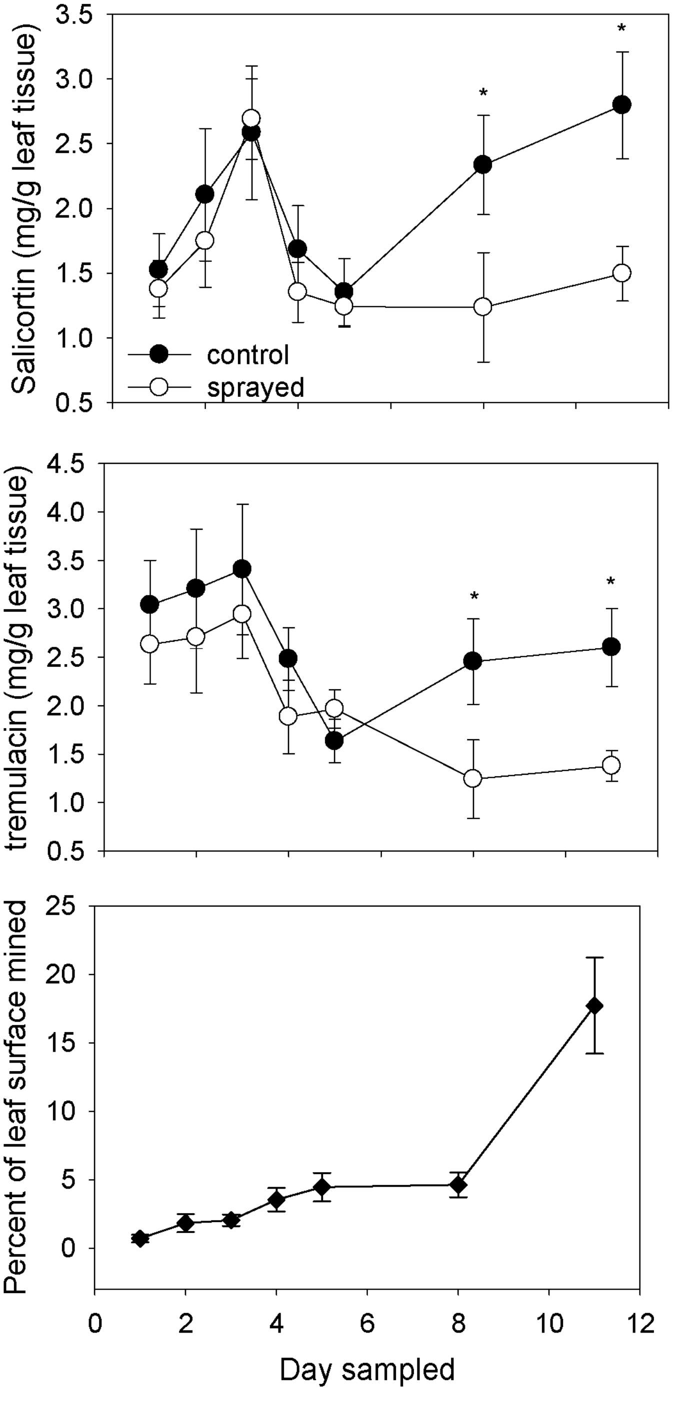

Fig. 2.4 Variation over time in (a) salicortin, (b) and tremulacin concentrations, and (c) percent naturally-mined (control) and insecticide-sprayed ramets during 2007. Values are means ± SE. * LS mean contrast, $P < 0.05$.

General conclusions

In the interior of Alaska, the leaves of *P. tremuloides* trees (<2.5 m in height) which bear EFNs contain higher concentrations of the PGs tremulacin and salicortin when compared to leaves without EFNs. This suggests that leaves within *P. tremuloides* trees vary considerably in the degree to which they are defended: a subset of leaves contain both high concentrations of chemical defenses and EFNs, which attract predacious arthropods. Epidermal leaf mining by *P. populiella* induces the concentrations of the PGs tremulacin and salicortin in *P. tremuloides* leaf tissue which in turn may increase the level of defense against this insect and insect herbivory which occurs later in the season. In my studies, the amount of leaf mining by *P. populiella* on *P. tremuloides* leaves was negatively, but weakly, related to the foliar concentrations of the PGs but was unrelated to the presence of EFNs. These findings shed new light on the relative distribution of two defenses within aspen trees, and suggest that chemical defense may contribute to patterns of herbivory previously attributed to EFNs alone.

There is still much to be discovered about the interactions of PGs and EFNs in *P. tremuloides* and their roles as forms of defense against the epidermal leaf miner *P. populiella.* Further investigations will be necessary to assess the impact of the predatory insect community on *P. populiella.* Lastly, additional studies will also be necessary to understand the effects of PGs on *P. populiella* development and performance.

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