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Extrafloral Nectaries in Aspen (*Populus tremuloides*): Heritable Genetic Variation and Herbivore-induced Expression

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• *Background and Aims* A wide variety of plants produce extrafloral nectaries (EFNs) that are visited by predatory arthropods. But very few studies have investigated the relationship between plant genetic variation and EFNs. The presence of foliar EFNs is highly variable among different aspen (*Populus tremuloides*) genotypes and the EFNs are visited by parasitic wasps and predatory flies. The aim here was to determine the heritability of EFNs among aspen genotypes and age classes, possible trade-offs between direct and indirect defences, EFN induction following herbivory, and the relationship between EFNs and predatory insects.
• *Methods* EFN density was quantified among aspen genotypes in Wisconsin on trees of different ages and broad-sense heritability from common garden trees was calculated. EFNs were also quantified in natural aspen stands in Utah. From the common garden trees foliar defensive chemical levels were quantified to evaluate their relationship with EFN density. A defoliation experiment was performed to determine if EFNs can be induced in response to herbivory. Finally, predatory arthropod abundance among aspen trees was quantified to determine the relationship between arthropod abundance and EFNs.
• *Key Results* Broad-sense heritability for expression (0·74–0·82) and induction (0·85) of EFNs was high. One-year-old trees had 20% greater EFN density than 4-year-old trees and more than 50% greater EFN density than $\geq$10-year-old trees. No trade-offs were found between foliar chemical concentrations and EFN density. Predatory fly abundance varied among aspen genotypes, but predatory arthropod abundance and average EFN density were not related.
• *Conclusions* Aspen extrafloral nectaries are strongly genetically determined and have the potential to respond rapidly to evolutionary forces. The pattern of EFN expression among different age classes of trees appears to follow predictions of optimal defence theory. The relationship between EFNs and predators likely varies in relation to multiple temporal and environmental factors.

Key words: Aspen, extrafloral nectaries, herbivory, indirect defence, induction, mutualism, optimal defence, *Populus tremuloides*, heritability, genetic variation.

INTRODUCTION

Extrafloral nectaries (EFNs) are the basis of important mutualisms between plants and ants in many systems (reviewed by Bentley, 1977; Koptur, 1992, 2005). In addition, parasitic wasps can be attracted at short range to EFNs (Stapel *et al.*, 1997; Röse *et al.*, 2006), which may translate into higher parasitism of insect herbivores (Pemberton and Lee, 1996). The attraction of predators to EFNs can provide protection to the plant (Cuautle and Rico-Gray, 2003; Ness, 2003; Kost and Heil, 2005; but see O'Dowd and Catchpole, 1983; Tempel, 1983; Rashbrook *et al.*, 1992), and has been demonstrated to be an indirect plant defence in some systems (Heil *et al.*, 2004).

Most of the studies cited above suggest a positive relationship between EFNs and predators (e.g. ants, wasps), and invoke an adaptive outcome of those relationships. However, EFNs must be heritable for the interactions to have an adaptive function (Mitchell, 2004). In the only study we know of that examines heritability of EFN traits, Rudgers (2004) found that EFN morphology (size) and density in wild cotton (*Gossypium thurberi*) were heritable. The lack of published research showing a heritable genetic basis for EFN characteristics is interesting, given the demonstrated importance of EFN–predator interactions in many systems over many years (Bentley, 1977; Koptur, 1992, 2005).

Patterns of EFN expression appear to follow predictions of optimal defence theory (McKey, 1974), being produced near particularly valuable tissues (e.g. flowers and fruits; Koptur, 1992), or young tissues (Heil *et al.*, 2000; Wäckers *et al.*, 2001; Mondor and Addicott, 2003). Presumably, producing EFNs in young tissues could increase the presence of herbivore natural enemies near those important plant parts. Although plant age affects other non-nectar extra-floral rewards (e.g. food bodies) and influences how ants, and potentially other predators, respond to those rewards (Fiala *et al.*, 1994; Nomura *et al.*, 2001; Del Val and Dirzo, 2003), to our knowledge no studies have demonstrated age-related variation in EFN density. Doak *et al.* (2007) showed that short ramets (0·5–2 m tall) had a greater EFN frequency than tall ramets and presumably short ramets were younger trees,

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but tree age was not reported. Because many plants induce greater nectar production or expression of EFNs after herbivory, the plant would be protected against herbivory when and where the defence is needed most (Koptur, 1989; Wäckers *et al.*, 2001; Heil *et al.*, 2001, 2004; Mondor and Addicott, 2003; Ness, 2003; Rogers *et al.*, 2003; Heil and Kost, 2006). In some studies, however, EFNs on younger tissues were inducible while EFNs on older tissues were not (Wäckers *et al.*, 2001).

Many plants that produce EFNs also have effective direct anti-herbivore defences, including trichomes (cotton: Rudgers *et al.*, 2004), thorns (*Acacia*: Huntzinger *et al.*, 2004), protective waxy coverings (*Macaranga* spp.: Federle *et al.*, 1997) and chemical defences (leguminous trees: Heil *et al.*, 2002). Direct and indirect defences (such as EFN-recruited predators) both require plant resources. Therefore, the existence of negative correlations (trade-offs) between direct and indirect defences is often hypothesized. Negative correlations are intuitively appealing and have been corroborated by some studies (e.g. Nomura *et al.*, 2001; Dyer *et al.*, 2003), but not others (Heil *et al.*, 2002; Del Val and Dirzo, 2003; Rudgers *et al.*, 2004). Rudgers *et al.* (2004) suggested that negative correlations between direct and indirect defences are more probably to be found when the indirect resistance trait is an obligate defence (e.g. EFNs in myrmecophytes) rather than a facultative defence.

Trembling aspen (*Populus tremuloides*) is an excellent model system to test for genetically based effects on EFNs because of its clonality and substantial genetic variation (Jelinski, 1993; Lindroth and Hwang, 1996). A relationship between *Populus* spp. EFNs and ants or parasitic wasps may have existed since the Oligocene (approx. 35 mya) (Pemberton, 1992) and clonal variation in aspen EFNs has been recently reported (Doak *et al.*, 2007).

Using aspen as the experimental system, five questions were addressed here. First, does aspen demonstrate heritable genetic variation in EFN density among genotypes? Addressing issues of heritable genetic variation in EFNs fills a gap in the literature (Mitchell, 2004) and more fully places EFNs into an evolutionary framework.

Second, does the distribution of EFNs follow predictions of optimal defence theory (McKey, 1974)? If EFNs are defensive, optimal defence theory would predict a greater proportion of EFNs on leaves of young (i.e. seedlings) trees or young tissues, compared with leaves on older trees or on older tissues because leaves on younger trees are proportionally more valuable than leaves on older trees.

Third, is the expression of EFNs induced by herbivory and is the capacity for EFN induction itself heritable? In several systems, herbivory resulted in induction of EFNs but no studies have demonstrated a genetic component to EFN induction.

Fourth, does a negative relationship between direct (chemical) and indirect (EFNs) defences exist? Because direct and indirect defences are potentially costly (Herms and Mattson, 1992) and redundant, trade-offs between them are predicted. In addition to condensed tannins, aspen produce the phenolic glycosides salicortin and tremulacin, which have been shown to influence herbivore performance (Hwang and Lindroth, 1997, 1998; Osier *et al.*, 2000; Donaldson and Lindroth, 2007).

Fifth, do trees with a higher density of EFNs attract more predatory (including parasitic) arthropods? We have observed parasitic flies (Tachinidae) and parasitic wasps (Ichneumonidae) feeding at aspen extrafloral nectaries. Both parasitic flies and wasps, as well as predaceous flies, attack major aspen defoliators and can control their populations at low herbivore densities (Parry *et al.*, 1997). However, the relationship between aspen EFNs and the abundance of predaceous arthropods is unknown.

MATERIALS AND METHODS

Trembling aspen (*Populus tremuloides* Michx.) trees were surveyed from three common gardens located in Wisconsin and from naturally occurring aspen stands at three sites in Utah. In Wisconsin, a long-term common garden was established at the Arlington Agricultural Experiment station, near Arlington. At different times, two other potted gardens were established on the University of Wisconsin-Madison (UW) campus. To distinguish among trees from each Wisconsin garden, the gardens will be referred to throughout the paper as 'Juvenile Common Garden', 'Juvenile Potted Garden' and 'Seedling Potted Garden'. Natural stands in Utah were in the Wasatch Mountains, located in the north-central part of the state.

The Juvenile Common Garden contains 15 ramets of 12 aspen genotypes collected from south-central Wisconsin and planted for long-term research. At the time of this study (2005) trees were 4 years old and 3–4 m tall. Genotypes, confirmed by microsatellite analysis (Cole 2005), were collected from Dane county (Dan1, Dan2), Pine Island (PI3, PI12), Parfrey's Glen (PG1, PG2, PG3), Sauk county (Sau1, Sau2, Sau3) and Waushara county (Wau1, Wau2). Trees were produced by tissue-culture micropropagation (Donaldson, 2005), and planted in 2002 as 1-year-old seedlings in a common garden at Arlington Agricultural Experiment Station, 30 km north of Madison, WI (43°16′20″N, 89°16′50″W). The garden was planted on a former agricultural field in a Plano Silt Loam soil.

The Seedling Potted Garden consisted of individuals from the same 12 aspen genotypes as above and was maintained outdoors on the UW campus. The trees in this garden were initially produced (2003) by tissue-culture micropropagation as above, and planted into 650-mL D40 Conetainers® (Stuewe and Sons, Inc., Corvallis, OR, USA). They were transplanted into 4-L pots containing 70% sand, 30% silt-loam soil in April, 2004, before leaf flush.

The Juvenile Potted Garden, also located on the UW campus, had eight aspen genotypes (a subset of the previous 12) and was established using genotypes replicated via micropropagation. In spring 2002 the 1-year-old micropropagated trees (average height = 1·1 m) were transplanted into 80-L pots containing a mixture of 70% sand and 30% silt-loam field soil.

To assess EFN density at a field site, putative aspen clones were surveyed at three sites in Utah in June, 2005.

The sites included: American Fork Canyon (AF), Aspen Grove (AG) and Big Springs (BS). Clones were selected within sites based on their spatial separation from other clones, growth form and leaf morphology (size and shape). Chemical analysis had previously been performed on these clones and they were found to be chemically distinct (Lindroth, Wooley and Donaldson, unpubl. res.). Four individuals were surveyed from each of five or six clones at each site (17 clones surveyed). Six clones were surveyed along the road through American Fork Canyon (40°26′50″N, 111°38′30″W to 40°25′52″N, 111°36′51″W). American Fork Canyon is on the west-south-west side of Mt Timpanogos and appears drier compared with the other two sites. On the eastern side of the mountain, five clones were surveyed at Aspen Grove (40°23′40″N, 111°34′59″W to 40°24′18″N, 111°36′22″W). At Big Springs (40°19′57″N, 111°31′30″W to 40°19′28″N, 111°31′55″) six clones were surveyed. Trees were between 2 and 6 m tall, appeared to be mature and displayed no evidence of significant herbivore damage.

Heritable genetic variation in EFNs

To examine the heritability of aspen EFNs, EFNs were quantified on 60 trees (4–6 trees for each of 12 genotypes) at the Juvenile Common Garden in July, 2005. Short shoots were examined on eight main branches from both the upper (four branches) and the lower (four branches) canopy of each tree. 'Short shoots' are the short branches arising from lateral buds along a main branch. They contain leaves formed the previous year (pre-formed leaves) and the number of leaves on short shoots does not change during the season. We assessed all leaves on four short shoots (1st, 3rd, 5th and 7th) (mean number of leaves surveyed: $13{\cdot}4 \pm 0{\cdot}4$, upper canopy; $6{\cdot}9 \pm 0{\cdot}1$, lower canopy) per branch, beginning at the most distal short shoot from the trunk and proceeding toward the trunk. Main branches were selected beginning with the first branch immediately below the main leader and working down from the top, and beginning with the branch closest to the ground and working up from the bottom. The vertical distance between the upper and lower canopies ranged from 1 to 1·5 m. A range of leaves (23–46) were censused on each ramet within and across genotypes. The number of leaves censused did not differ significantly among ramets ($P = 0{\cdot}148$) or among genotypes ($P = 0{\cdot}360$). Natural stands of aspen in Utah were surveyed for EFNs using the same methodology as the surveys in Wisconsin but the distance between the upper and lower canopy was greater and ranged from 1 to 2·5 m.

The term "EFN density" is used here to describe the proportion of leaves with EFNs (number of leaves with EFNs/total number of leaves examined). We summed across shoots and calculated EFN density for the upper and lower canopies separately. Overall EFN density for a tree was also calculated [(upper canopy EFN density + lower canopy EFN density)/2]. Overall EFN density values are reported when comparing EFN density among genotypes from the Juvenile Common Garden and among clones from the Utah EFN surveys.

EFNs were quantified on leaves from trees of the Seedling Potted Garden to determine heritability of EFNs on young trees. Trees were censused in July, 2004 after growing in the 4-L pots for 3 months. Trees had not yet developed lateral branches, were approx. 1 m tall and 1 year old. Therefore, all leaves were examined for the presence/absence of EFNs on each of five trees from 12 genotypes (60 total trees). The leaves examined were neo-formed leaves.

Herbivory-induced expression of EFNs

In the Juvenile Potted Garden, the effect of defoliation on EFN induction was assessed as measured by changes in EFN density between defoliation treatments. The eight genotypes were replicated over five blocks and either defoliated or not defoliated. The defoliation treatment occurred in early June, 2002 and 2003 and was designed to simulate an insect outbreak in both duration and intensity. Randomly selected trees in the defoliation treatment were damaged using both forest tent caterpillars (*Malacosoma disstria*) and scissors. In each year a subset (1–2) of branches on each tree was bagged and several third-instar caterpillars were introduced into each bag. Feeding by insects may provide cues that are important for induction (Karban and Baldwin, 1997; Havill and Raffa, 1999). The bulk of the defoliation was subsequently accomplished using scissors to remove 75% of each leaf, thereby ensuring that each tree received the same amount of damage (Stevens *et al.*, 2007). After two seasons of defoliation when trees were 3 years old, EFNs were censused (2004) using the same methods as used at the Juvenile Common Garden.

Relationship between direct and indirect defences

To determine if a negative relationship between direct (chemical) and indirect (EFN) defences existed, leaves for chemical analysis were collected from the Juvenile Common Garden at nearly the same time EFNs were assessed. Leaves from all 60 trees were collected from throughout the canopy to represent foliar chemistry of the entire tree. Leaves were returned to the laboratory and flash-frozen in liquid nitrogen, freeze-dried and ground on a Wiley Mill (#40 mesh screen). From these samples, phenolic glycosides (PG) and condensed tannins (CT) were extracted and quantified. The phenolic glycosides salicortin and tremulacin were quantified by high-performance thin layer chromatography (Lindroth *et al.*, 1993), using purified salicortin and tremulacin as reference standards. Condensed tannins were extracted with 70% acetone and quantified using the butanol-HCl method of Porter *et al.* (1986). Aspen tannins were purified (Hagerman and Butler, 1994) and used as a reference standard.

Predatory insects and EFNs

In the Juvenile Common Garden, insect predators were censused to determine the differences in the number of

predatory arthropods among aspen genotypes. The relationship between predators and EFNs was also evaluated. In July, 2005 both sides of bright yellow index cards (3 × 5″) were coated with a thin layer of clear Tangletrap® paste (The Tanglefoot Company, Grand Rapids, MI, USA) and placed within the canopy of each aspen tree assessed for EFNs. Cards were orientated similarly and placed at the same height (1·5 m) in each of the sampled canopies. Sticky cards were left in the canopy for 3 d, and then returned to the laboratory. A dissecting microscope was used to identify and count predaceous arthropods (wasps, flies, spiders and ants) on the cards. Both predatory flies (Dolichopodidae) and parasitic flies (Tachinidae) were grouped into one category and the data reported as predaceous flies.

Statistics

Similar statistical analyses were performed for EFN data from the Wisconsin common and potted gardens and Utah field sites. Transformations were made as needed to normalize EFN data and insect count data. All analyses were performed using JMP 5·0·1 (SAS Institute, Inc., 2002). Means are presented ± 1 s.e. in the text and $+1$ s.e in the figures.

Heritable genetic variation in EFNs. Average EFN density was compared among aspen genotypes using an ANOVA model with aspen genotype as a fixed effect. The same model was used to test for differences in EFN density among genotypes planted in the Seedling Potted Garden. For the clones surveyed in Utah, a nested ANOVA model was used to compare average EFN density among sites and clones. Model variables were site, clone nested within site, and tree nested within both site and clone as a random variable. Including the replicate (tree) nested within site and clone as a random variable provides the correct denominator degrees of freedom for testing the effects of site and clone on EFN density. An interaction term (site × clone) was not included because clones were not replicated among sites. Average EFN density among clones was also compared within each site (three separate tests) using an ANOVA model with EFN density as the response and clone as the main effect. Finally, a simple correlation analysis of mean EFN frequency among all three common gardens was performed.

Broad-sense heritability (H^2_B) was calculated for average EFN density in the Juvenile Common Garden and in the Seedling Potted Garden. H^2_B was also calculated for the induction response. Measures of broad-sense heritability ('degree of genetic determination'; Falconer, 1989) estimate the genotypic contribution to the phenotype. Broad-sense heritability is calculated by dividing the total genetic variation, V_G (an estimate of additive genetic variance plus all other forms of genetic variance), by the total phenotypic variance, V_P, for all trees. Calculations of V_G and V_P from ANOVA results were made as described in Falconer (1989). We are aware that measures of H^2_B likely overestimate evolutionary potential (Mitchell, 2004) and interpret our results accordingly. High values (>0·5) of H^2_B are suggestive of a strong/rapid response to some selective force(s) (Mitchell, 2004). Values of H^2_B are given $\pm$95% CI. Upper and lower 95% confidence limits were calculated by $H^2_B \pm 1{\cdot}96 \times$ s.e.

Distribution of EFNs within the canopy. Using data from the Juvenile Common Garden, a nested mixed-model ANOVA was performed to determine if EFN density differed between upper or lower canopy positions within and among aspen genotypes. EFN density was the dependent variable, with genotype and canopy position as fixed main effects and genotype × canopy position as the interaction term. Tree nested within genotype was a random variable to provide the correct denominator degrees of freedom for testing the effect of genotype on EFN density. The same ANOVA model was used to compare within-tree EFN density among clones in Utah. To compare differences between the upper and lower canopy within a genotype a paired *t*-test was performed because measures of upper and lower canopy EFN density were not considered independent measures (Zar, 1999).

Herbivory-induced expression of EFNs. EFN density was compared between experimental (defoliated) and control (non-defoliated) trees from the Juvenile Potted Garden using a factorial ANOVA with defoliation and genotype as fixed effects and genotype × defoliation as the interaction term. The analysis was performed with and without block in the model. Block was not significant ($P > 0{\cdot}14$) in any analysis and so we report results only from the factorial analysis with block removed. An increase in EFN density in the defoliated trees compared with non-defoliated trees was considered an induced response. Paired *t*-tests were performed to determine if the defoliation-induced change in EFN density was significant within a genotype.

Relationship between direct and indirect defences. Leaves for chemical analysis were collected from only the Juvenile Common Garden. After quantifying foliar concentrations, concentrations of foliar defensive chemicals (condensed tannins, salicortin and tremulacin) were regressed against the average EFN density among aspen genotypes.

Predatory insects and EFNs. Data from sticky trap counts from trees at the Juvenile Common Garden were normalized and compared among aspen genotypes via a one-way ANOVA. To determine the relationship between EFN density and predatory arthropods, regression analysis was performed with average EFN density as the predictor and counts of each group of interest (parasitic wasp, predatory flies, spiders and ants) as the response variable. Average EFN density for each tree was used rather than comparisons among either upper or lower canopy measures of EFNs because sticky cards were placed midway between the upper and lower canopy.

RESULTS

Heritable genetic variation in EFNs

Among the 12 aspen genotypes examined, EFN density was highly variable in both juvenile and seedling trees. Among

genotypes at the Juvenile Common Garden, average EFN density ranged from 23 to 65%, with an mean density of 46·4 ± 2% (Fig. 1A). Variation in EFN density among genotypes in the Seedling Common Garden ranged from 30 to 100%, with a mean density of 64·3 ± 2·6% (Fig. 1B). Broad-sense heritability values for EFN density ranged from 0·82 ± 0·15 at the Juvenile Common Garden to 0·74 ± 0·17 at the Seedling Common Garden.

Correlations of the average EFN density among trees in the different gardens mirrored the differences in age among those trees. The EFN density of the Juvenile Potted Garden was most strongly correlated with that of the Juvenile Common Garden ($r = 0{\cdot}68$, d.f. = 8 $P = 0{\cdot}06$). In turn, the EFN density of the Juvenile Potted Garden was most strongly correlated with that of the Juvenile Common Garden ($r = 0{\cdot}62$, d.f. = 8, $P = 0{\cdot}10$). The weakest correlation was found between the EFN density of the youngest trees (Seedling Common Garden) and the oldest trees (Juvenile Common Garden) ($r = 0{\cdot}42$, d.f. = 12, $P = 0{\cdot}16$).

Overall, EFN density was more than 50% lower among clones surveyed at field sites in Utah compared with genotypes in Wisconsin. Average EFN density among Utah clones ranged from 3·6 to 45%, with a mean density of 20·3 ± 1·3% (Fig. 2). Average EFN density was not different among Utah sites (AG, 22·5 ± 3·01%; BS, 21·2 ± 3·06%; AF, 17·9 ± 0·79%; $F_{2,64} = 0{\cdot}35$, $P = 0{\cdot}70$). However, average density was different within sites at Aspen Grove and Big Springs, but not at American Fork Canyon (Fig. 2).

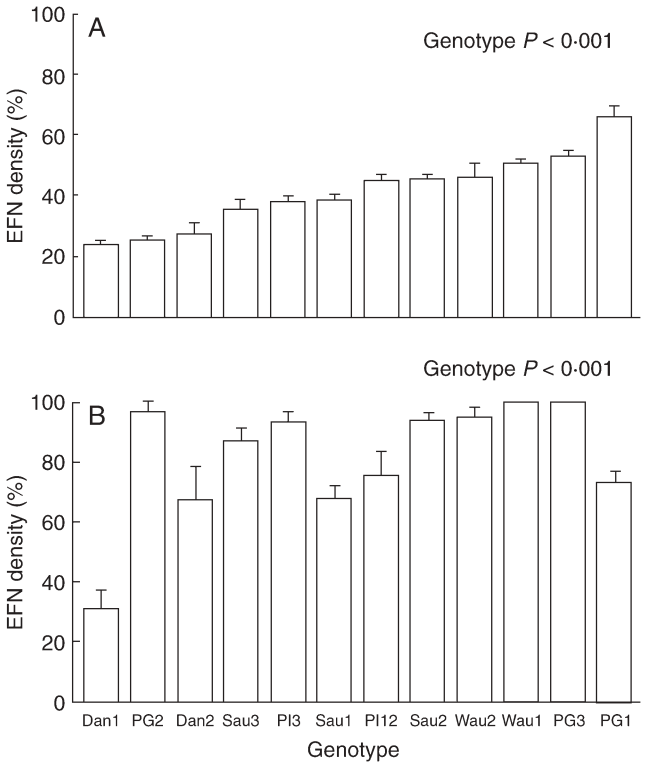

FIG. 1. (A) EFN density on aspen genotypes from the Juvenile Common Garden. (B) EFN density on 1-year-old aspen trees in the Seedling Common Garden. Bars indicate means +1 s.e. ($n = 5$). Genotypes (e.g. PG3 and Wau1) with EFNs on every leaf had no variance about the mean in the 1-year-old aspen trees. Bars are ordered from lowest to highest based on the EFN density at the Juvenile Common Garden.

Distribution within the canopy

EFN density varied significantly between canopy positions within Wisconsin trees. In general, the upper canopy had 44% more EFNs than the lower canopy, but a difference in EFN density between upper and lower canopy positions occurred in all but one genotype (Wau2). The genotype × canopy position interaction (Fig. 3) likely resulted from the large variation among genotypes in the magnitude of the difference in EFN density between canopy positions. In contrast, EFN density was only marginally higher in the upper canopy than in the lower canopy in trees from Utah ($F_{1,47} = 3{\cdot}59$, $P = 0{\cdot}064$). EFN density between upper and lower canopies was similar within all clones (non-significant clone × canopy position interaction) ($F_{16,47} = 1{\cdot}56$, $P = 0{\cdot}117$).

Herbivory-induced expression of EFNs

Across all genotypes, defoliated trees had a 23% greater EFN density than non-defoliated trees ($F_{1,78} = 59{\cdot}56$, $P < 0{\cdot}001$). In some genotypes, EFN density nearly doubled, while other genotypes (e.g. Dan1, Wau1) showed no significant induction response (significant genotype × defoliation interaction; Fig. 4). Broad-sense heritability for the induction response was 0·85 ± 0·11, indicating a large genetic component for herbivory-induced EFN expression.

Relationship between direct and indirect defences

Average EFN density on trees from the Juvenile Common Garden was not associated with foliar levels of condensed tannins ($F_{1,54} = 0{\cdot}68$, $P = 0{\cdot}41$, $R^2 = 0{\cdot}01$), salicortin ($F_{1,54} = 0{\cdot}05$, $P = 0{\cdot}81$, $R^2 = 0{\cdot}001$) or tremulacin ($F_{1,54} = 1{\cdot}5$, $P = 0{\cdot}22$, $R^2 = 0{\cdot}02$). These results suggest that no trade-off exists between direct (chemical) and indirect (EFN) defence in aspen.

Predatory insects and EFNs

In the Juvenile Common Garden, parasitic wasp ($F_{11,59} = 0{\cdot}78$, $P = 0{\cdot}65$), spider ($F_{11,59} = 1{\cdot}4$, $P = 0{\cdot}19$) and ant ($F_{11,59} = 0{\cdot}99$, $P = 0{\cdot}46$) abundance did not differ among aspen genotypes, nor did they differ in relation to average EFN density. In contrast, predatory fly abundance varied significantly among aspen genotypes (Fig. 5). However, no relationship was found between average EFN density and the number of predaceous flies ($F_{1,59} = 1{\cdot}2$, $P = 0{\cdot}27$, $r^2 = 0{\cdot}02$) or any other predaceous arthropod.

DISCUSSION

Heritable genetic variation

This study is one of only a few to demonstrate intraspecific variation in an EFN trait, and the first to demonstrate a

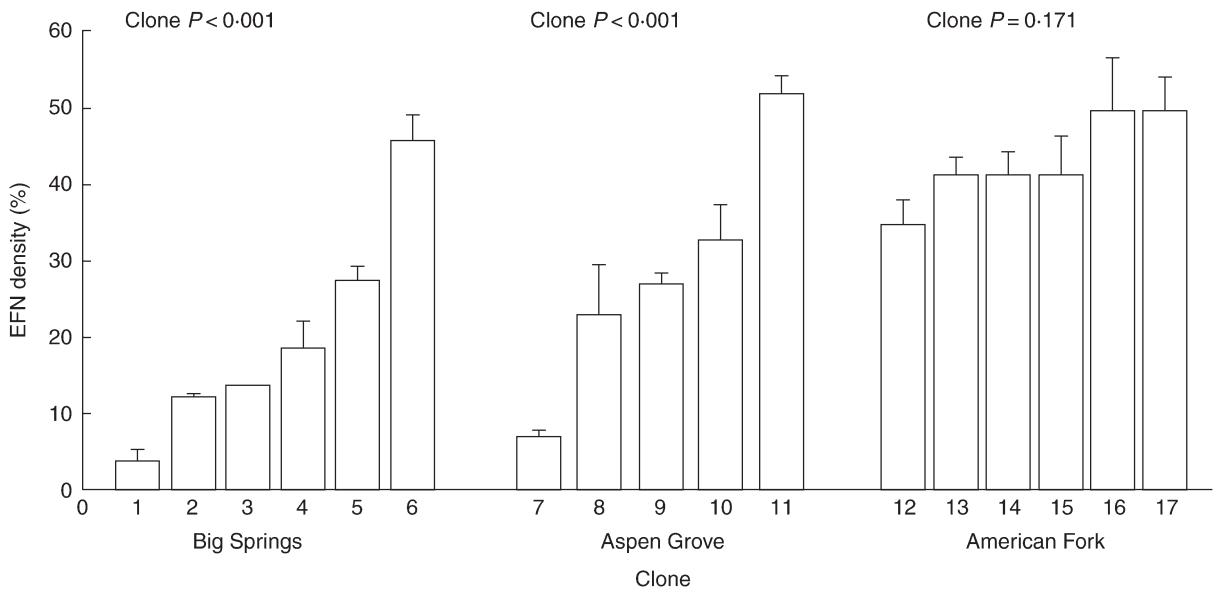

FIG. 2. EFN density among natural aspen stands in Utah. Trees were >10 years old. Bars indicate means +1 s.e. ($n = 4$). We use the term 'clone' instead of genotype because we do not have genetic data to confirm clones were distinct genotypes.

heritable genetic basis to EFN expression in a tree species. Because EFN density is a highly heritable trait, aspen have the potential to respond rapidly to factors selecting for EFN expression. We recognize that broad-sense heritability overestimates evolutionary potential. However, we believe the pattern of marked variation in EFN expression demonstrated in this study indicates strong evolutionary potential because of the experimental design employed. Replicated genotypes grown randomly in a common garden were used, thereby eliminating environmental variance as much as possible.

The presence of many more EFNs on younger trees than on older trees indicates that expression of aspen EFNs follows the pattern predicted by optimal defence theory (McKey, 1974), similar to findings for extrafloral nectar in cotton (Wäckers and Bonifay, 2004). Leaves of young trees are of great photosynthetic value for two reasons. First, because 1-year-old trees have relatively few leaves, each leaf has a higher value to the plant compared with each leaf on older, larger trees. Second, aspen are very sensitive to light levels (Osier and Lindroth, 2006) and must grow quickly to compete for available light. Therefore,

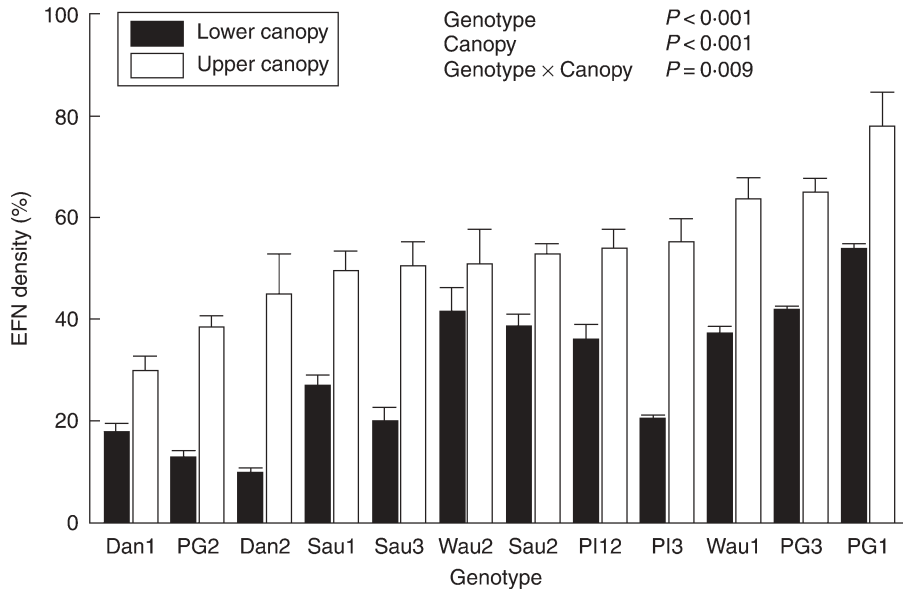

FIG. 3. Within-canopy variation in EFN density on aspen genotypes from the Juvenile Common Garden. Bars are ordered from lowest to highest density based on upper canopy density. Bars indicate means +1 s.e. ($n = 5$).

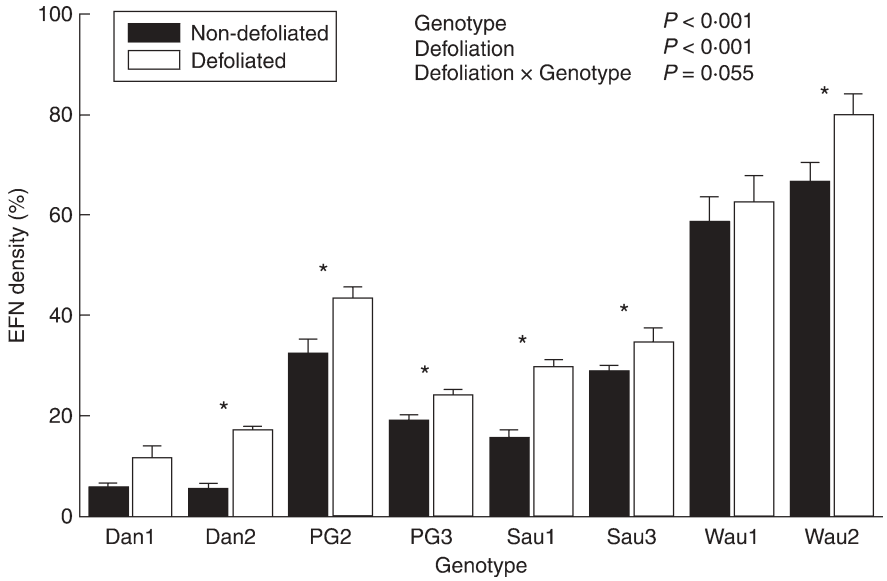

FIG. 4. EFN density compared among defoliated (light bars) and non-defoliated (dark bars) aspen genotypes in the Juvenile Potted Garden. Defoliated trees were exposed to two seasons of defoliation while non-defoliated did not receive a damage treatment. Bars indicate +1 s.e. ($n = 5$). Asterisks (*) indicate differences significant at $P < 0{\cdot}05$.

leaves on young trees are predicted to be more highly defended than leaves on older trees because each leaf contributes a greater proportion of photosynthate to the tree than does each leaf on an older tree. Aspen EFN density seems to follow this pattern. Among genotypes in Wisconsin, 1-year-old trees had EFNs on 64% of their leaves, 3-year-old trees had EFNs on 51% of their leaves and 4-year-old trees had EFNs on 46% of their leaves. In addition, the correlation of mean EFN density becomes weaker as the age between trees increases. For example, mean EFN density of 1-year-old trees is more highly correlated with 3-year-old trees than with 4-year-old trees. We recognize that comparison of the potted common garden trees with the planted common garden trees in Wisconsin confounds age with location. Nonetheless, the results, especially those comparing potted common garden trees, suggest that expression of EFNs is probably under both strong ontogenetic and genetic control, as is expression of other aspen traits (e.g. phytochemistry; Donaldson *et al.*, 2006). Similarly, Doak *et al.* (2007) suggest that EFN expression is strongly determined by developmental stage.

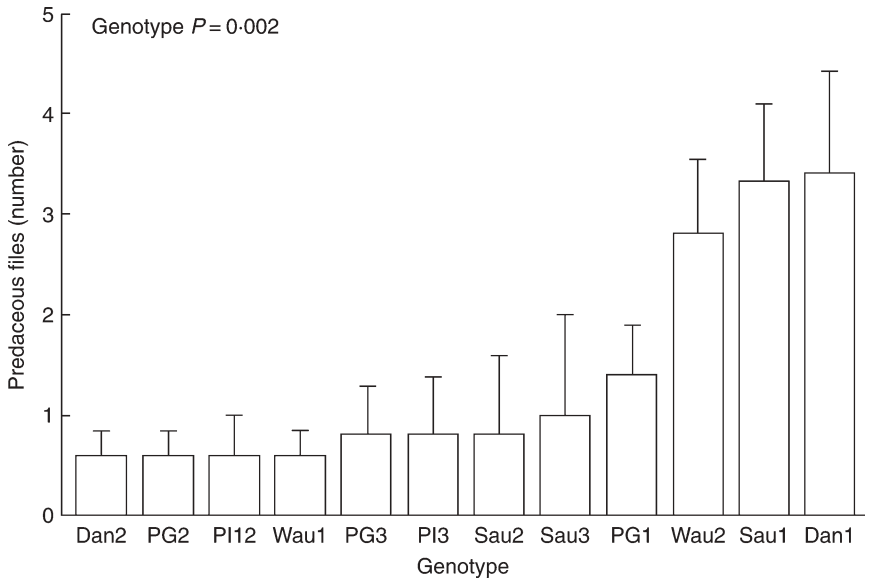

FIG. 5. Predaceous fly abundance among aspen genotypes in the Juvenile Common Garden. Fly abundance values were obtained from a sticky trap census. Bars indicate +1 s.e. ($n = 5$).

Distribution within the canopy

That leaves in the upper canopy had more EFNs than leaves in the lower canopy may be explained by physiological factors. In the case of aspen, upper leaves are likely not light-limited compared with lower canopy leaves. Therefore, the cost of producing EFNs in the upper canopy may be less than the cost of producing EFNs in the lower canopy. Alternatively, leaves in the upper canopy may be more valuable in terms of photosynthate production and therefore worthy of greater investment in protection.

An alternative hypothesis for the greater presence of EFNs in the upper canopy compared with the lower canopy is that EFNs are outlets for excess carbohydrate (reviewed in Bentley, 1977; Koptur, 2005). The fact that insects feed on the extrafloral nectar may be incidental to the presence of EFNs. Indeed, leaves in the upper canopy are probably not light-limited and therefore may produce excess carbohydrate. However, the vast majority of published results do not support such a hypothesis (Koptur, 2005). Rather, the most convincing case for the presence of EFNs in most systems is that they help in protecting the plant. In the case of aspen, within-tree variation in EFN density may not result from a single cause, but instead, from the interaction of physiological costs of producing EFNs and the benefit of recruiting predators of herbivores, influenced by different environments (see Doak *et al.*, 2007).

EFN induction

Overall, EFN density was 23% higher among trees that suffered two seasons of defoliation, compared with non-defoliated trees. Similarly, Mondor and Addicott (2003) found that defoliation in broad beans resulted in an increase of 59–106% more EFNs, with greater amounts of defoliation resulting in increased induction. Although no published studies have shown an increase in EFN density after defoliation in trees, Ness (2003) found that EFN secretion increased in *Catalpa bignonioides* trees after herbivory, consequently recruiting more ants to the leaves that were fed upon. Rogers *et al.* (2003) reported similar results for extrafloral nectar induction on Chinese tallow tree (*Sapium sebiferum*).

Because the induction response is heritable, *P. tremuloides* may respond to herbivore-driven directional selection with increased EFN expression. Selective pressures in aspen could include expansive, multi-year outbreaks of lepidopteran defoliators (e.g. forest tent caterpillars). These defoliators are targeted by specialist and generalist tachinid (Parry *et al.*, 2003; Stireman *et al.*, 2006) and sarcophagid (Parry *et al.*, 1997) flies. In our system, we have observed tachinids feeding on aspen EFNs, suggesting the potential for EFNs to play a role in herbivore predation by flies. Ants may also play a role in defending aspen in established aspen stands. Currently, ants are not present in substantial numbers in our 4-year-old common garden but may play a larger role in older stands.

Relationship between direct and indirect defences

Much of the study of aspen defence has focused on chemical factors (Lindroth, 2001; Donaldson *et al.*, 2006; Osier and Lindroth, 2006; Donaldson and Lindroth, 2007; Stevens *et al.*, 2007; Wooley *et al.*, 2007). However, indirect defences such as EFNs may play a role in conjunction with anti-herbivore chemicals. Because resources are limited, trade-offs between direct and indirect defences may exist. In the present system, no trade-off between EFN density and anti-herbivore chemicals was detected. Similarly, there were no trade-offs found between herbivore resistance and tolerance in aspen (Stevens *et al.*, 2007). In other words, at least for the variables measured here, aspen could potentially defend itself against herbivory both directly (chemically) and indirectly (EFN-recruited predators). The present results join a number of other published studies showing no negative relationship between direct and indirect defences (Brody and Karban, 1992; Thaler and Karban, 1997; Underwood *et al.*, 2000). The lack of a trade-off between EFNs and chemical defences lends support to the hypothesis of Rudgers *et al.* (2004) that because the EFN–predatory arthropod relationship in aspen is not an obligate relationship, trade-offs are not probably to be found.

Insect predators of herbivores and EFNs

The current results are consistent with the few published reports of no effect of EFNs on predatory insect abundance (O'Dowd and Catchpole, 1983; Tempel, 1983; Rashbrook *et al.*, 1992). Among the predatory arthropods collected from the aspen trees in our study, parasitic wasps, ants and spiders were not attracted to particular aspen genotypes nor were their abundances related to EFN density. In contrast, predaceous fly abundance did vary among genotypes, but that variation was not related to EFN density. However, EFNs may influence the amount of time predatory arthropods remain in the aspen canopy without increasing the number of arthropods caught on sticky cards if predatory arthropods walk, rather than fly, among the leaves.

These results beg the question: why do aspen produce EFNs if they are not attractive to herbivore predators? We offer three possibilities. First, parasitoids may be attracted more by herbivore cues than by EFN density because EFNs appear to be attractive only at close distances (Röse *et al.*, 2006). For example, over long distances, parasitoids are probably attracted more by volatile cues from the plants and herbivore frass (Mondor and Roland, 1997) than by the presence of EFNs. However, the presence of EFNs could influence predatory arthropod search time within the canopy. When our work was conducted, herbivore densities were low (especially compared with outbreak densities) and may simply not have been high enough to attract parasitoids, especially density-dependent parasitoids (Roland and Taylor, 1997).

Second, predator abundance at EFN-bearing plants is context dependent (Tempel, 1983). For example, the abundance of some tachinids is influenced by forest structure, with small fragments having fewer tachinids (Roland and

Taylor, 1997). Our common garden is a part of a forest fragment among agricultural fields, which may have reduced the abundance of tachinids.

Third, EFNs may be maintained by periods of intense selection (e.g. insect outbreaks) (O'Dowd and Catchpole, 1983; Rudgers and Strauss, 2004) when direct defences (chemicals) are incapable of reducing outbreak populations (Donaldson, 2005). In the declining years of outbreak conditions, parasitoids are important in reducing herbivore numbers (Roland and Taylor, 1997; Stireman *et al.*, 2006). At the same time, trees that were attacked in previous years may have an increased EFN density (induction). Thus, trees with a greater EFN density may survive outbreaks more often than trees with lower EFN density because of the differential attraction of parasitoids.

CONCLUSIONS

This is one of the first studies to demonstrate heritability of EFNs, and the first to show heritability of herbivore-induced EFN expression. Our results, coupled with those of Doak *et al.* (2007), demonstrate that expression of EFNs has a genetic basis across geographically widespread sites (Wisconsin to Utah to Alaska). We echo Mitchell's (2004) suggestion that more effort be given to studies of the genetic basis of EFN traits, to place EFN biology more firmly into an evolutionary context. In addition, because variation in EFN expression between different age classes occurs in tropical (e.g. *Macaranga* spp.; Fiala *et al.*, 1994) and temperate (Doak *et al.*, 2007; this study) trees, age-related variation in EFN traits in other plant species may be widespread. Furthermore, EFNs may not always be costly in terms of a reduction in allocation to defensive chemicals. Instead, the presence of both chemical (direct defence) and EFNs (indirect defence) may be important for effective defence across a broad range of herbivory events (e.g. endemic to outbreak conditions).

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