Oecologia
DOI 10.1007/s00442-010-1799-6

PLANT-ANIMAL INTERACTIONS - ORIGINAL PAPER

# Defensive effects of extrafloral nectaries in quaking aspen differ with scale

**Brent Mortensen · Diane Wagner · Patricia Doak**

Received: 16 April 2010 / Accepted: 14 September 2010

**Abstract** The effects of plant defenses on herbivory can differ among spatial scales. This may be particularly common with indirect defenses, such as extrafloral nectaries (EFNs), that attract predatory arthropods and are dependent on predator distribution, abundance, and behavior. We tested the defensive effects of EFNs in quaking aspen (*Populus tremuloides* Michx.) against damage by a specialist herbivore, the aspen leaf miner (*Phyllocnistis populiella* Cham.), at the scale of individual leaves and entire ramets (i.e., stems). Experiments excluding crawling arthropods revealed that the effects of aspen EFNs differed at the leaf and ramet scales. Crawling predators caused similar reductions in the percent leaf area mined on individual leaves with and without EFNs. However, the extent to which crawling predators increased leaf miner mortality and, consequently, reduced mining damage increased with EFN expression at the ramet scale. Thus, aspen EFNs provided a diffuse defense, reducing damage to leaves across a ramet regardless of leaf-scale EFN expression. We detected lower leaf miner damage and survival unassociated with crawling predators on EFN-bearing leaves, suggesting that direct defenses (e.g., chemical defenses) were stronger on leaves with than without EFNs. Greater direct defenses on EFN-bearing leaves may reduce the probability of losing these leaves and thus weakening ramet-scale EFN defense. Aspen growth was not related to EFN expression or the presence of crawling predators over the course of a single season. Different effects of aspen EFNs at the leaf and ramet scales suggest that future studies may benefit from examining indirect defenses simultaneously at multiple scales.

**Keywords** Plant–animal interactions · Tritrophic · Biotic defense · Scaling · Defense hypothesis

## Introduction

Interactions between species produce outcomes which may be viewed at a range of spatial scales; however, the perceived strength and outcome of these interactions may differ with scale (reviewed by Addicott et al. 1987; Levin 1992). In the case of plant–herbivore interactions, the influence of defenses expressed on a part of a plant may extend to neighboring tissue (e.g., Shelton 2005; Shroff et al. 2008). For example, in cotton (*Gossypium* spp.), cavities containing high concentrations of defensive chemicals provide defense to surrounding, relatively undefended, tissue (reviewed by Rudgers et al. 2004). Thus, vegetation within the same "ecological neighborhood" (Addicott et al. 1987) may share a similar level of defense regardless of branch or leaf-scale traits. As a result, a defensive trait may appear ineffective at fine spatial scales, such as leaves or shoots, while reducing herbivore damage at larger scales, for example, over an entire plant.

---

Communicated by Colin Orians.

**Electronic supplementary material** The online version of this article (doi:10.1007/s00442-010-1799-6) contains supplementary material, which is available to authorized users.

B. Mortensen · D. Wagner (✉) · P. Doak
Department of Biology and Wildlife,
Institute of Arctic Biology,
University of Alaska Fairbanks,
Fairbanks, AK 99775-7000, USA
e-mail: diane.wagner@alaska.edu

*Present Address:*
B. Mortensen
Department of Ecology, Evolution,
and Organismal Biology, Iowa State University,
Ames, IA 50011, USA

The issue of scale may be of particular importance when examining the effects of indirect defenses. Indirect defenses are a common mechanism whereby plants attract predators to reduce damage by herbivores (reviewed by Heil 2008); however, individual predators make foraging decisions at multiple spatial scales. For example, predators may selectively forage on plants based on the density of a single, favored species of prey (i.e., inter-plant scale) but consume multiple species after arriving on a plant (i.e., intra-plant scale) (Östman and Ives 2003).

Scaling effects on indirect defense may be common in the case of extrafloral nectaries (EFNs), nectar secreting structures not typically associated with reproduction (Bentley 1977). By providing food rewards, EFNs attract predators such as ants (Bentley 1977; Heil 2008), parasitoids (Rose et al. 2006), and mites (Pemberton 1993) capable of disrupting herbivore feeding. EFNs can influence predators at the intra- and inter-plant scales, for example, by attracting predators to damaged portions of a single plant (Ness 2003) or luring predators onto a plant (Rudgers 2004). Reponses by predators to EFNs may also occur simultaneously at both scales as on senita cacti (*Pachycereus schotti* Engelmann) where EFNs deter ants from visiting flowers at the intra-plant scale while increasing ant abundance at the inter-plant scale (Chamberlain and Holland 2008). However, few studies have explicitly examined the effects of EFNs on herbivory simultaneously at multiple scales (but see Rudgers and Strauss 2004; Doak et al. 2007).

Quaking aspen (*Populus tremuloides* Michx.) is particularly suitable for examining the effects of EFNs on plant defense within and across clonal stems, or ramets, from one or more genetic individuals, or genets. In aspen, EFNs appear on a subset of leaves, with most leaves having either 0 or 2 EFNs on the adaxial (top) surface near the petiole (Doak et al. 2007). The percentage of leaves with EFNs on a ramet (hereafter, EFN frequency) can vary both within and across stands (Doak et al. 2007; Wooley et al. 2007; Online Resource 1). Natural variation of EFN expression in aspen at the leaf and ramet scales offers the possibility of examining the scale(s) at which EFNs operate in relation to plant defense.

Aspen EFNs may serve a defensive role against herbivores. Early descriptions of aspen EFNs coincide with observations of visitation by predatory arthropods (Trelease 1881). A more recent study has reported a negative relationship between damage by a specialist herbivore, the aspen leaf miner (*Phyllocnistis populiella* Cham., hereafter, ALM), and the presence of EFNs at the leaf scale and EFN frequency at the shoot (i.e., branch and associated leaves produced in the current growing season) scale (Doak et al. 2007). However, the occurrence of EFNs on aspen leaves is positively correlated with phenolic glycoside concentrations which, when found above a threshold concentration, are negatively correlated with ALM damage (Young et al. 2010). Thus, in the case of aspen, the possible effects of EFNs and phenolic glycosides on herbivory are confounded. Experimental approaches, such as excluding predators from plants, are necessary in distinguishing the effects of these two putative defenses.

We experimentally assessed whether aspen EFNs reduce ALM damage by attracting predatory arthropods at the leaf and ramet scales. As the effects of EFNs may depend on their ecological context (e.g., predator or herbivore abundance; Barton 1986; Rudgers and Strauss 2004), we assessed the generality of potential relationships by conducting research at nine aspen stands in interior Alaska, USA. We tested three main predictions by experimentally excluded crawling predators from a subset of aspen ramets at each site:

1. EFNs mediate an indirect defense by crawling predators in quaking aspen at the leaf and ramet scales. While EFNs on an individual leaf may encourage predators to forage locally, reducing mining damage on the target leaf (i.e., leaf-scale “targeted defense”), EFNs may impact herbivory at larger scales if predators forage on neighboring leaves while traveling to or from an EFN, reducing ALM damage over entire ramets as EFN frequency increases (i.e., ramet-scale “diffuse defense”).
2. Crawling predators reduce the density of ALM larvae, and thus mining damage, by decreasing egg densities or increasing egg and larval mortality in proportion to EFN expression.
3. EFNs convey net benefits to aspen ramets in the form of increased growth.

## Materials and methods

### Study system

The aspen leaf miner (*Phyllocnistis populiella* Cham., ALM), a univoltine, gracillariid moth, has been at outbreak levels in interior Alaska since 2001 (US Forest Service 2008) and in portions of the Yukon Territory, Canada since the early 1990s (Canada Natural Resources 2008). Overwintering adults emerge in late April to early May and oviposition coincides with aspen bud break in early to mid-May, extending for 2–3 weeks (Condrashoff 1964). Eggs sink into the leaf epidermis and larvae hatch in 1–2 weeks. Larvae feed on the leaf epidermis for 2–3 weeks with prognathous mouthparts that prevent movement through the mesophyll or out of the leaf (Condrashoff 1962); thus, individuals on one side of the leaf are isolated from those on the other surface. Intraspecific competition can be severe, and contact between larvae can result in one larva killing the other (Condrashoff 1964). After feeding, larvae pupate in leaf folds, eclosing 2 weeks later in late June to early July. Damage by the ALM reduces aspen growth (Wagner et al. 2008) and may be associated with aspen dieback during prolonged outbreaks (Canada Natural Resources 2008).

ALM eggs, larvae, and pupae are subject to predation by arthropods known to visit EFNs, including ants, mites, and parasitoid wasps (personal observation). Ants prey on ALM eggs, larvae, and pupae, removing larvae and pupae from mines and leaf folds, respectively. Ants found on aspen at our study sites include *Formica dakotensis* Emery, *F. neorufibarbis* Emery, *F. subsericea* group, *Camponotus herculeanus* L., *Leptothorax* sp. (new species near *muscorum*), and *Myrmica brevispinosa* Wheeler (determinations by G. Alpert and S. Cover, Museum of Comparative Zoology, Harvard University). Mites (*Balaustium* sp.) prey on ALM eggs and puncture larvae to feed on the hemolymph through the leaf cuticle, killing the larvae while leaving carcasses intact with little evidence of attack (personal observation). Voucher specimens for ants and mites observed in this study are available in the University of Alaska Museum in Fairbanks. Idiobiont parasitoid wasps (including six species of eulophids; Doak et al. 2007; P. Doak and D. Wagner, unpublished data) attack both larvae and pupae, arresting feeding and development in immature ALMs.

### Experimental design

We experimentally excluded crawling predators from aspen ramets at nine sites in interior Alaska, near Fairbanks (USA, 64°49′N, 147°59′W; Online Resource 1) during the summer of 2007. Sites were located on the edge of mature aspen clones. EFN expression varied among sites with 33–79% of all leaves collected in mid-June bearing EFNs (Online Resource 1). Sites were chosen over a 45 km × 10 km area to avoid overlap of aspen clones among sites (mean distance to nearest site: 3.2 km; range: 0.08–10.3 km). At each site, we haphazardly selected 32–34 ramets 0.5–3 m in height spaced over an area of $\sim$125 m$^2$.

Before bud break at each site, we excluded crawling arthropods from 15–17 haphazardly chosen ramets $\geq$3 m tall by circling the base of the trunk with a band of sticky gel (Tangle-Trap; Tanglefoot, Grand Rapids, MI, USA) over a layer of flexible plastic. We maintained the exclusion treatment through the end of the growing season, marked by leaf fall in early September, so as to measure the effects of EFNs and crawling predators on seasonal ramet growth. As necessary, we removed neighboring vegetation to prevent bridging over the barrier. Unmanipulated ramets served as controls.

### Do EFNs reduce mining damage at the leaf and ramet scales?

If crawling predators reduce ALM damage and are attracted by EFNs, then (1) mining damage will be greater on exclusion than control ramets, and (2) the difference in mining damage between treatments will increase with EFN expression (exclusion × EFN effect). We measured the percent leaf area mined (hereafter, mining damage) on leaves from two shoots per ramet (9–16 leaves per ramet). We selected shoots haphazardly, rejecting shoots with severe, non-ALM damage (>50% of leaf area missing). Shoots were harvested in early to mid-June after most ALM larvae had pupated (i.e., ceased feeding), but adults had not yet eclosed, and stored in separate plastic bags at 5°C until measured. Although mining damage is negatively correlated with leaf longevity, leaves with extensive damage (76–100% of the leaf surface mined) do not abscise on average until early September (Wagner et al. 2008). Thus, there is little likelihood that leaves collected in mid-June were biased towards leaves with low ALM damage. We measured the percent area mined on leaf tops and bottoms using visual estimates. Visual estimates of each observer were strongly correlated with damage measured using imaging analysis software (Scion Image, Fredrick, Maryland; $r^2 \geq 0.9$, $P < 0.001$; Doak et al. 2007).

We recorded EFN expression for each leaf as present or absent. The proportion of leaves with EFNs on a ramet (EFN frequency) may change through the growing season with the addition of late-formed leaves (Doak et al. 2007). Therefore, in the case of models testing oviposition and egg predation, we calculated EFN frequency based on leaves that were present on selected shoots during egg surveys, when oviposition and egg predation occurred. As late-formed leaves may reduce mining damage on older leaves via predator attraction, we calculated EFN frequency for herbivory and growth models based on all leaves produced on harvested shoots. EFNs occurring on leaves that had abscised prior to harvesting may have also influenced mining damage at the ramet scale. Therefore, we determined EFN expression on missing leaves using data collected during egg counts.

We examined the effects of crawling predator exclusion (hereafter, exclusion), EFN expression, leaf side (top, bottom), and their interactions on the percent area mined per leaf surface using ANCOVA. We differentiated between leaf sides as ALM damage differs between leaf tops and bottoms, particularly in relation to EFN expression (Doak et al. 2007). Herbivory, oviposition, and predation may each vary depending on the relative position of a leaf on a shoot. Therefore, we included leaf position as a covariate, numbering leaves from the base of the shoot continuing outwards, accounting for missing leaves by recording the presence of leaf scars. Leaves that had abscised between shoot collection and measurement were not included for analysis as their original position could not be determined. For all models described in this paper, we limited our response variables to leaf positions 1–8 as leaves beyond position 8 were typically absent during ALM oviposition.

### Do crawling predators reduce ALM density in response to EFNs?

In order for crawling predators to reduce mining damage by decreasing ALM survival, mining damage must decline with reduced ALM abundance and survival. However, earlier work suggests that, depending on leaf size, the percent area mined on a leaf surface may reach a maximum with as few as one (personal observation) or two (Condrashoff 1964) ALM larvae present. Furthermore, a leaf may receive many more ALM eggs than is required to completely mine its surface, particularly during outbreak conditions (Condrashoff 1964). Thus, impacts of crawling predators on ALM density will only lead to substantial declines in damage if (1) mining damage is related to ALM abundance below some threshold density of larvae, and (2) predators reduce larval density below this threshold value.

We tested for a threshold effect in the relationship between mining damage and ALM larval density using piecewise linear regression (PROC NLIN, SAS 9.1; SAS Institute, Cary, NC, USA). The model included the effects of the number of larvae that survived to finish feeding, leaf side, and their interaction. As more larvae may be necessary to maximize damage on larger leaves, we included leaf width, a correlate of leaf area ($r^2 = 0.94$, $n = 42$, $P < 0.001$), as a covariate. By using a piecewise regression model, we were able to estimate the point at which damage ceased to increase with increasing ALM survival. We tested the relationship between ALM density and damage above and below this threshold value using ANCOVA, including density and leaf side with their interaction as fixed effects and leaf width and position as covariates. We compared the threshold model to a linear model using the Akaike information criterion (AIC). The linear model included the same parameters but assumed a linear relationship between damage and ALM density. We limited our analysis of mining damage and ALM density to control ramets to determine the threshold at which mining damage is maximized under natural conditions.

We tested whether crawling predators reduced ALM density below the threshold necessary to affect mining damage on individual leaves. We compared treatments in terms of the proportion of leaf surfaces with ALM densities above and below the threshold using a chi-squared test. If the density of surviving larvae was below the threshold on a significantly greater portion of control than exclusion leaf surfaces, then crawling predators may reduce mining damage by increasing ALM mortality.

While the above models test whether or not crawling predators can reduce ALM damage, they do not indicate if this relationship is influenced by EFN expression. We tested the effects of EFNs on two possible mechanisms by which crawling predators may reduce ALM density and, therefore, ALM damage.

First, crawling predators may reduce the density of ALM eggs laid on a leaf, promoting a subsequent reduction in mining damage. We measured ALM egg density at each site near the conclusion of oviposition, 12–19 days after bud break. We counted the number of ALM eggs, initiated mines, and marks left from eggs that had been removed (hereafter "egg scars"), summing these values to estimate initial egg density. Because eggs removed within several hours of oviposition are unlikely to leave a mark, our measure of egg density is a function of both oviposition and early egg predation. Shoots surveyed for egg abundance were tagged and used for mining estimates and one leaf per shoot was marked with a tag on the petiole to aid identification of individual leaves. We examined the effects of exclusion, EFN expression, leaf side, and their interactions, with leaf position as a covariate, on the number of eggs present per leaf surface.

Second, crawling predators may reduce mining damage by increasing ALM egg and larval mortality. We recorded egg mortality as the presence of egg scars during egg counts. Larval mortality was measured on leaves harvested for mining estimates by opening mines to determine whether larvae had completed feeding and, if applicable, the cause of death. We classified cause of death as parasitized, removed, or unexplained. Parasitized ALM larvae were identified by the presence of a parasitoid egg, larva, or pupa on or near the dead ALM. We defined removal as cases where a mine had been opened and the larva removed. Unexplained death included all cases where we found larvae dead within the mine but could not determine the cause of death. Unexplained death includes, but is not necessarily limited to, predation by mites or adult parasitoid wasps, intraspecific competition, and possibly death due to chemical defenses.

We examined the effects of exclusion, EFN expression, leaf side, and their interactions on ALM mortality at the scale of individual eggs and larvae using mixed model logistic regression (PROC GLIMMIX, SAS 9.2; SAS Institute, Cary, NC, USA). We limited our analyses to mortality occurring before larvae completed feeding as mortality can only affect damage during this period. Leaf position was included as a covariate in both models. As intraspecific competition may have contributed to larval mortality, the number of ALM larvae on a leaf surface was also included as a covariate in the larval mortality model.

To better understand the cause(s) of larval mortality, we tested the effects of crawling predators and EFN expression on whether a larva was removed, parasitized, or died from unexplained causes. We describe these tests and their results in Online Resource 2.

### Do EFNs confer benefits to aspen ramets in the form of increased growth?

We tested the effect of ALM damage on two forms of growth for control ramets. At the end of the 2007 growing season, we measured the three longest current-year shoots on each ramet (Wagner et al. 2008). In 2008, we collected cross-sections of the central trunk of each ramet and measured the 2006 and 2007 radial growth rings approximately 0.2 m above ground level using a sliding bench micrometer with a precision of 0.01 mm. For each ramet, measurements were made along two orthogonal axes, and the values were averaged. Ramets with rings that could not be reliably assigned to a specific growing season were excluded from analyses.

To better account for other factors that may affect ramet growth, we measured the approximate canopy cover and vegetation density around each ramet. We measured canopy cover by photographing the canopy above each ramet. In each instance, we positioned the camera directly above the ramet facing south at a vertical angle equal to the mean, noontime declination of the sun during the growing season. We converted photos into binary graphics using ImageJ v. 1.38 (Abramoff et al. 2004) to calculate the percent cover. Vegetation density was measured as the number of plants from any species taller than 0.5 m within a 1.5-m radius of each ramet.

Mining damage has different physiological effects on aspen depending on the leaf side affected (Wagner et al. 2008). As damage between sides is related ($r^2 = 0.266$, $t_{4,504} = 40.43$, $P < 0.001$), we tested the effect of mining on each leaf side in separate ANCOVA models to avoid issues of colinearity. Ramet growth was measured as mean maximum shoot length or mean radial growth. We used mean radial growth from the previous year, percent canopy cover, and vegetation density as covariates in all growth models.

If EFNs mediate an indirect defense which promotes ramet growth, then growth will increase with EFN frequency more rapidly when crawling predators are present than absent. We examined the effects of exclusion, ramet EFN frequency, and their interaction on radial growth using ANCOVA. EFN frequency almost certainly depends on shoot length as leaves formed late in the growing season have high rates of EFN expression (Doak et al. 2007). Therefore, we examined the effect of exclusion, but not EFN expression, on mean maximum shoot length.

### Statistics: general considerations

We ran mixed model ANCOVAs using the random maximum likelihood (REML) method (JMP 8; SAS Institute, Cary, NC, USA), calculating denominator degrees of freedom using the Kenward–Roger method (Kenward and Roger 1997). Depending on the scale of reference, we included leaf, shoot, ramet, and site as random effects. We examined the distributions of residual values with normal probability plots to test whether model assumptions were met and used natural log transformations as necessary. In the case of significant interactions between fixed effects in the models described above, we tested individual slopes or means using a Student's *t* test or Tukey's comparison, respectively.

## Results

### Do EFNs reduce mining damage at the leaf and ramet scales?

Crawling predators reduced mining damage by 22% ($P < 0.001$; Table 1); however, the effect of crawling predators in relation to EFN expression differed at the leaf and ramet scales. At the leaf scale, leaves with EFNs received 4% less mining damage than leaves without EFNs ($P < 0.001$); however, this relationship was similar between exclusion and control ramets (EFN × exclusion: $P = 0.387$; Table 1, Fig. 1a). The lack of a significant interaction suggests that crawling predators do not explain a significant portion of the variation between leaves with and without EFNs. In contrast, the negative effect of crawling predators on mean ALM damage rose significantly with ramet EFN frequency (ramet EFN × exclusion: $P = 0.012$; Table 1, Fig. 1b).

On average, leaf tops received 8% more mining damage than leaf bottoms ($P < 0.001$). Crawling predators had a greater impact on mining damage on leaf bottoms, reducing damage by 24% compared to 20% on leaf tops ($P = 0.001$; Table 1).

### Do crawling predators reduce ALM density in response to EFNs?

Mining damage increased significantly with the number of ALM larvae that completed feeding per leaf surface in both the piecewise and simple linear regression models (threshold model: $r^2 = 0.78$, $P < 0.001$; linear model: $r^2 = 0.75$, $P < 0.001$). However, piecewise regression, which assumed that the relationship between the density of surviving larvae and damage differed above and below a threshold value, provided a better fit than a simplified model assuming a

**Table 1** Effects of crawling predators and EFN expression on aspen leaf miner (*Phyllocnistis populiella*) damage and mortality

| Source | Mining damage | | | Egg removal | | Larval mortality | |
|---|---|---|---|---|---|---|---|
| | *F* | Den. DF | *P* | $F_{123,943}$ | *P* | $F_{17,896}$ | *P* |
| EFN[a] | **12.76** | **3,309.0** | **<0.001** | 0.11 | 0.741 | **9.63** | **0.002** |
| Side | **127.09** | **3,648.0** | **<0.001** | **6.96** | **0.008** | 0.86 | 0.354 |
| EFN × side | 3.05 | 3,647.0 | 0.081 | 0.00 | 0.972 | 0.70 | 0.403 |
| Ramet EFN frequency[b] | 0.56 | 299.3 | 0.456 | 1.43 | 0.231 | 1.06 | 0.304 |
| Ramet EFN × side | 0.07 | 3,647.0 | 0.797 | 0.18 | 0.671 | 0.44 | 0.506 |
| Exclusion | **114.83** | **284.7** | **<0.001** | 0.37 | 0.544 | 0.16 | 0.685 |
| EFN × exclusion | 0.75 | 3,256.0 | 0.387 | 0.08 | 0.779 | 0.00 | 0.994 |
| Side × exclusion | **11.49** | **3,648.0** | **0.001** | 1.00 | 0.316 | 0.68 | 0.409 |
| EFN × side × exclusion | 1.28 | 3,647.0 | 0.259 | **3.96** | **0.047** | 0.08 | 0.781 |
| Ramet EFN × exclusion | **6.46** | **312.5** | **0.012** | **11.07** | **0.001** | 2.59 | 0.108 |
| Ramet EFN × side × exclusion | <0.01 | 3,647.0 | 0.959 | 1.19 | 0.275 | 1.86 | 0.172 |
| Leaf position[c] | 0.60 | 3,279.0 | 0.438 | **87.13** | **<0.001** | **21.13** | **<0.001** |
| N (ALM larvae present) | – | – | – | – | – | **251.94** | **<0.001** |

Effects were determined using a mixed model ANCOVA for damage and logistic regression for egg and larval mortality. Values shown in bold are significant at $\alpha = 0.05$

[a] Presence or absence of EFNs at the leaf scale

[b] Proportion of leaves on a ramet with EFNs. For the model of egg removal, EFN frequency was calculated for only those leaves present when eggs were counted; for models of larval mortality, this value was calculated using all leaves present when shoots were harvested

[c] Position of a leaf along a shoot, beginning at the base and continuing outwards

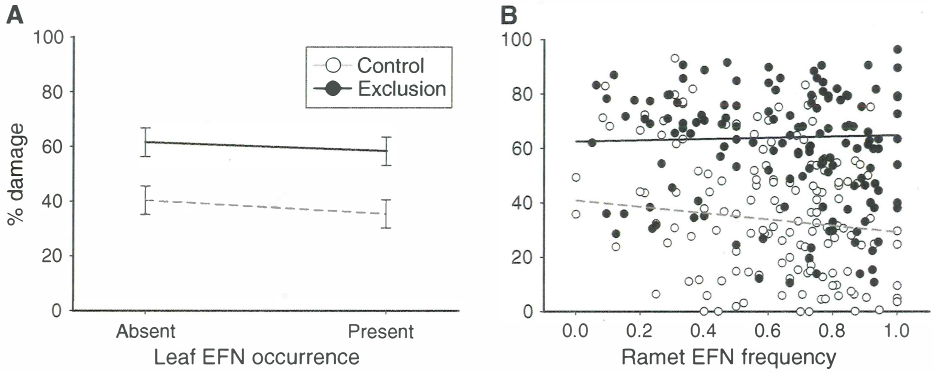

**Fig. 1** Percent leaf area mined by the aspen leaf miner (*Phyllocnistis populiella*) in relation to EFN expression at the **a** leaf and **b** ramet scales in the presence (*open points, dashed, gray lines*) and absence (*closed points, black lines*) of crawling predators ($n = 7{,}308$ leaf surfaces). Differences between treatments represent the effects of crawling predators in relation to EFN expression. Leaf-scale values are represented by least square means ± SE

constant relationship (threshold model: AIC = 33,268.07; linear model: AIC = 33,811.27; Fig. 2a). Therefore, we report results only from the piecewise regression model.

On average, the percent area damaged per leaf surface increased by 52.30 ± 0.96% with each larva that completed feeding ($t_{3,536.68} = 54.57$, $P < 0.001$) up to 1.16 ± 0.10 larvae. Above this threshold density, damage did not significantly increase with additional surviving larvae ($t_{3,538.65} = 1.26$, $P = 0.209$; Fig. 2a). The proportion of leaf surfaces with ALM densities below this threshold was significantly greater on control than exclusion ramets ($X^2_1 = 36.96$, $P < 0.001$; Fig. 2b).

Crawling predators reduced initial egg densities by 0.37 ± 0.02 (11%) eggs per leaf surface ($F_{1,285.3} = 6.75$, $P = 0.010$); however, initial egg density was unrelated to EFN expression at the leaf or ramet scales regardless of exclusion ($P > 0.05$). In the absence of crawling predators, we recorded 4.34 ± 0.11 (mean ± SE) eggs per leaf surface (the sum of eggs, egg scars, and mines; median = 2) with 89% of all surveyed leaf surfaces receiving at least one egg. Leaf tops received 0.50 ± 0.004 (24%) more eggs than leaf bottoms ($F_{1,3605} = 301.29$, $P < 0.001$).

The effect of crawling predators on ALM survival differed between the exposed (egg) and enclosed (larval)

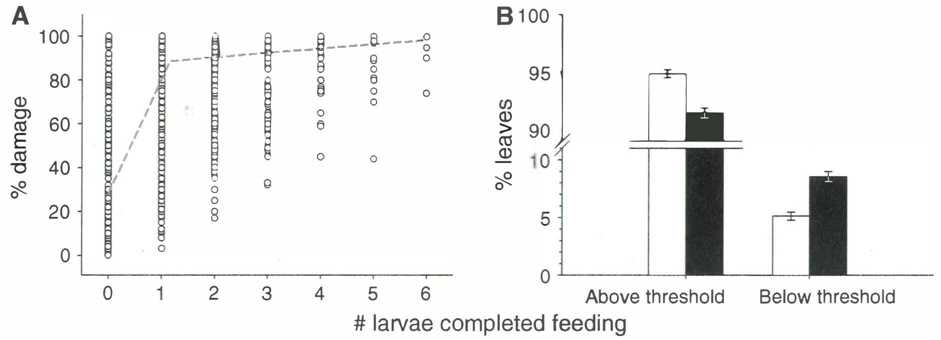

**Fig. 2** Indirect effects of crawling predators on ALM damage via changes in ALM larval density. **a** Percent area mined per leaf surface in relation to the number of ALM larvae that completed feeding on leaf positions 1–8 ($n = 3{,}615$ leaf surfaces). The slope of the piecewise regression line is significant before, but not after, the threshold at 1.16 larvae ($\alpha = 0.05$). ALM mortality caused by predators that reduced ALM density below this threshold would, on average, decrease leaf mining damage. **b** Proportion of leaf surfaces with densities of surviving larvae above or below the threshold shown in (**a**) for predator exclusion (*closed bars*) and control (*open bars*) leaf surfaces ± SE ($n = 8{,}046$ leaf surfaces)

life stages. Based on the frequency of egg scars on leaves, crawling predators increased the likelihood that an ALM egg was removed from 7.76 ± 0.21% on exclusion ramets to 18.73 ± 0.34% on control ramets, a 141% increase. While the main exclusion effect was not significant, we found a significant interaction among exclusion, the presence of EFNs on a leaf, and leaf side ($P = 0.047$; Table 1). Pairwise comparisons controlling for the presence of EFNs and leaf side revealed that egg removal was consistently lower on exclusion leaves ($P < 0.05$; Fig. 3a), suggesting that the significance of the exclusion effect was absorbed by this three-way interaction. Eggs were 38.9% more likely to be removed from leaf bottoms than leaf tops ($P = 0.008$; Table 1, Fig. 3a).

The effects of EFN expression on the relationship between crawling predators and egg removal differed between the leaf and ramet scales (Table 1, Fig. 3). At the leaf scale, the effect of crawling predators on egg removal was similar on leaves with and without EFNs ($P = 0.779$; Table 1, Fig. 3a). However, crawling predators were more likely to remove an egg as ramet EFN frequency increased, counteracting a negative relationship between ramet EFN frequency and removal on exclusion ramets ($P = 0.001$; Table 1, Fig. 3b).

Despite their effects on egg mortality, we found no evidence that crawling predators significantly impacted ALM larval mortality regardless of EFN expression (Table 1). Across treatments, 61.78 ± 0.47% of all larvae died while feeding, with 53.32 ± 0.48% of all larvae dying from unexplained causes. Parasitism affected 7.58 ± 0.25% of all larvae, while only 0.89 ± 0.09% of larvae were removed from the mine. Predator exclusion did not significantly affect ALM mortality ($P = 0.685$); however, ALM larvae were 7% more likely to die on leaves with EFNs than leaves

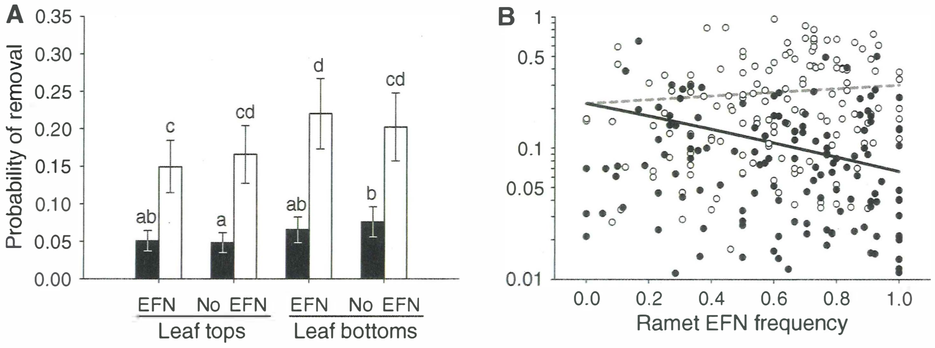

**Fig. 3** Probability that an ALM egg was removed in relation to EFN expression ($n = 27{,}289$ eggs). **a** Leaf-scale effects. The probability of egg removal in the presence (*open bars*) and absence (*closed bars*) of crawling predators. Mean values represent least square means ± SE from the logistic regression model described in the text. *Different letters* represent significantly different values based on Tukey's multiple comparison at $\alpha = 0.05$. **b** Ramet-scale effects. The relationship between probability of egg removal and ramet EFN frequency in the presence (*closed points*, *black line*) and absence (*open points*, *dashed*, *gray line*) of crawling predators. Equations for lines were determined using logistic regression and slopes were tested using $t$ tests (control: $t_{23,943} = 0.89$, $P = 0.372$; exclusion: $t_{23,943} = -2.96$, $P = 0.003$)

lacking EFNs on both exclusion and control ramets ($P = 0.002$). Larval mortality increased with larval density and leaf position ($P < 0.001$; Table 1).

### Do EFNs confer benefits to aspen ramets in the form of increased growth?

Mean radial growth of aspen ramets declined by 1% as the mean area damaged on leaf tops increased from 0 to 100% ($F_{1,90.72} = 3.97$, $P = 0.049$) but was not significantly related to damage on leaf bottoms ($F_{1,73.25} = 0.77$, $P = 0.383$). Shoot growth was not significantly related to ALM damage on leaf tops or bottoms (top: $F_{1,99.73} = 0.38$, $P = 0.540$; bottom: $F_{1,92.97} = 0.08$, $P = 0.776$). Ramet growth was not significantly affected by crawling predators (radial: $F_{1,237.9} = 0.04$, $P = 0.848$; shoot: $F_{1,239.9} = 3.27$, $P = 0.072$), irrespective of ramet EFN frequency (radial: $F_{1,237.1} = 1.94$, $P = 0.165$).

## Discussion

### Do EFNs reduce mining damage at the leaf and ramet scales?

Whereas crawling predators reduced damage by the aspen leaf miner at both the leaf and ramet scales, EFNs only mediated this effect at the ramet scale (Table 1, Fig. 1). The effect of EFNs at the ramet but not leaf scale can be explained by a diffuse, rather than targeted, EFN defense. If predator foraging is not targeted to leaves with EFNs, as we have shown at the leaf scale, then crawling predators influenced by EFNs at the ramet scale exert equal effects on leaves with and without EFNs. Thus, as more predators are attracted to or retained on ramets with greater EFN frequencies, the effects of crawling predators on ALM damage may increase while remaining similar among leaves with and without EFNs on the same ramet. A similar response was shown for *Catalpa bignonioides* Walter, where increased sugar production from EFNs on damaged leaves led to increased ant attendance on both damaged and undamaged leaves (Ness 2003).

By limiting observation to a single scale, researchers may overlook important interactions in relation to EFNs and other indirect defenses. Meta-analysis has shown that apparent effects of ant–plant mutualisms can depend on the scale of replication (Chamberlain and Holland 2009). For example, if EFNs operate at the plant scale, as in this study, experiments excluding predators from portions of plants would be unlikely to alter herbivory on plants with low EFN availability. Effects of scale may also occur across communities or landscapes in which experiments are conducted, as EFNs can influence site selection by predators in relation to foraging or nest formation (Bestelmeyer 2005; Wagner and Nicklen 2010). Thus, EFNs on individual plants or plant parts may be less effective at reducing damage in sites with low EFN availability.

While crawling predators did not target individual leaves based on the presence of EFNs, leaves with EFNs had significantly less damage than leaves without EFNs (Table 1, Fig. 1a). It is possible that the reduction in mining damage associated with EFNs was caused by flying predators that were not excluded by our experimental treatment. However, although parasitoids feed from aspen EFNs (personal observation), the probability that an ALM larva was parasitized was not related to the occurrence of EFNs on the host leaf (Online Resource 2). As no other flying predators have been observed attacking the ALM, we find it unlikely that flying predators are responsible for reductions in ALM damage associated with EFNs at the leaf scale.

Alternatively, reductions in ALM damage on leaves with EFNs may be the result of correlated defensive traits such as phenolic glycosides (Young et al. 2010). This conclusion is supported by our finding that unexplained death, which may include mortality caused by chemical defenses, was greater on leaves with EFNs (Online Resource 2). Thus, reductions in ALM damage on EFN-bearing leaves are more likely a primary result of a direct defense, chemical or otherwise, rather than an indirect, EFN-mediated defense.

In the case of aspen, diffuse EFN defense may help explain increased concentrations of phenolic glycosides in EFN-bearing leaves (Young et al. 2010). The cost of losing a leaf without EFNs may be confined to the loss of a single leaf. However, if a ramet loses a leaf with EFNs, the cost extends beyond the loss of a single leaf to the potential loss of neighboring tissue that would have benefited from EFN-mediated defense. By increasing the concentration of phenolics in leaves with EFNs (Young et al. 2010), aspen may reduce the likelihood of weakening or losing a diffuse defense. The possibility that plants maintain defenses at one scale (e.g., individual leaves) so as to prevent the degradation of defenses at other scales (e.g., shoot, plant) may help clarify some instances wherein direct and indirect defenses are not negatively correlated according to traditional "trade-off" hypotheses (Heil et al. 2002; Wooley et al. 2007).

Diffuse defense is likely to decrease the cost–benefit ratio of EFNs relative to targeted defense. Ecological costs of EFNs have been identified (Adjei-Maafo et al. 1983; Heil et al. 2004; Rudgers and Strauss 2004), and suggestive evidence exists for direct costs of EFN production and maintenance (Adjei-Maafo et al. 1983; Rudgers 2004; Rutter and Rausher 2004; Sugiura et al. 2006). Counteracting these costs, in many cases, EFNs benefit plants by reducing herbivore damage as shown by this and other studies (reviewed by Bentley 1977; Heil 2008). If the benefits of

EFNs extend to neighboring leaves, then a subset of a plant's leaves may receive the benefits of indirect defense without incurring the cost of EFN expression. This may help explain notable variation in EFN expression within aspen and other species such as horsebean (*Vicia faba* L.; Mondor and Addicott 2003), whistling thorn (*Acacia drepanolobium* Sjost.; Huntzinger et al. 2004), and wild cotton (*Gossypium thurberi* Todaro; Rudgers 2004).

Whether defense is diffuse or targeted, some "optimal," intermediate level of EFN expression may exist that minimizes the cost to benefit ratio of EFNs over an entire plant. This "optimal" EFN frequency will not be constant across time and space as the relative costs and benefits of EFNs depend on environmental conditions, including the identities and abundances of herbivores and predators (Barton 1986; Di Giusto et al. 2001; Rudgers and Strauss 2004). Phenotypic plasticity of aspen EFN frequency through induction in response to herbivore pressure (Wooley et al. 2007) may allow genets to approximate this optimum under different conditions.

### Do crawling predators reduce ALM density in response to EFNs?

Crawling predators decreased the number of ALM larvae that completed feeding on each leaf by reducing initial egg densities and increasing ALM mortality. While both mechanisms contributed to reductions in ALM damage (Fig. 2), increased ALM mortality was the only effect of crawling predators related to EFN expression.

Crawling predators had the greatest effect on ALM mortality during the exposed egg rather than the enclosed larval life stage. Although the cause of the negative relationship between egg removal and ramet EFN frequency in the absence of crawling predators is unknown, crawling predators were more likely to remove ALM eggs from ramets with greater EFN frequencies (Fig. 3b). By comparison, EFN expression at the leaf scale did not influence egg removal by crawling predators (Fig. 3a). Thus, patterns of egg predation support our findings that EFNs mediate a diffuse, rather than targeted, defense against ALM damage on individual leaves. This finding contributes to earlier work showing that predators can reduce herbivory on a plant by increasing herbivore mortality in response to EFN expression (Bentley 1977; Di Giusto et al. 2001).

Crawling predators did not influence larval mortality regardless of leaf- or ramet-scale EFN expression. By feeding internally, the ALM may have some degree of protection from predation (but see Connor and Taverner 1997). For example, on bracken fern (*Pteridium aquilinum* L.), ants attracted by EFNs did not attack herbivores within mines or galls, but readily attacked these same herbivores when removed from their protective structures (Heads and Lawton 1985). Thus, crawling predators attracted to aspen EFNs may provide a greater defense against external than internal feeders.

Crawling predators further reduced ALM damage, though not in proportion to EFN expression, by decreasing the initial number of eggs present on a leaf. Two non-exclusive mechanisms may support this pattern. First, herbivores can be deterred from ovipositing on plants based on the presence of ants (Sendoya et al. 2009) and mites (Nomikou et al. 2003), possibly increasing adult and/or offspring survival. Furthermore, crawling predators may directly harass or attack adult ALMs in the 2–6 min required to lay a single egg after arriving on a leaf (Condrashoff 1964). Second, differences in egg density may be caused by egg predation shortly after oviposition. Eggs removed within several hours of oviposition typically do not leave a mark (personal observation) and could not be accounted for during egg counts. As crawling predators removed ALM eggs (Fig. 3), it is likely that, by extension, early predation reduced the initial number of eggs recorded on control ramets.

### Do EFNs confer benefits to aspen ramets in the form of increased growth?

Reductions in ALM damage by crawling predators did not translate into increased aspen growth, nor was growth significantly related to EFN expression. Although radial growth decreased slightly as ALM damage increased from 0 to 100% on leaf tops, crawling predators reduced ALM damage on leaf tops by only 19%, an effect evidently too small to increase ramet growth. By comparison, an insecticide treatment leading to 80–90% reductions in ALM damage increased both radial and shoot growth on aspen ramets (Wagner et al. 2008). Thus, the relatively small effects of crawling predators on ALM damage may be insufficient to produce noticeable differences in aspen growth over a single growing season.

Crawling predators may still be capable of mediating increases in aspen growth under different conditions. As previously mentioned, EFNs may have a greater impact on externally feeding herbivores than on the internally feeding ALM. If so, EFNs may promote aspen growth by limiting other forms of herbivory.

## Conclusions

While showing no response to EFNs at the leaf scale, crawling predators reduced ALM damage in proportion to EFN expression at the ramet scale by removing ALM eggs. This coarse scale of response suggests that EFN defense in aspen is diffuse rather than targeted to individual leaves with EFNs. Few studies have simultaneously examined the effects of EFNs at multiple scales (but see Ness 2003; Rudgers and Strauss 2004; Doak et al. 2007; Chamberlain and Holland 2008), possibly contributing to variable outcomes in studies of EFN defense (Bronstein 1998). We submit that future research may benefit from examining the effects of indirect defenses at multiple scales and locations in unison.

**Acknowledgments** Many thanks are extended to S. Fischer, T. Fristoe, I. Lien, T. Miner, and S. Wilbur for their invaluable assistance in data collection; D. Sikes for assistance identifications and comments on an earlier version of this paper; G. Alpert and S. Cover for ant determinations; H. Proctor for mite determinations; M. Short and J. McIntyre for guidance with statistics; B. Young for insightful conversation; and L. Mortensen. This paper was improved thanks to extremely helpful comments made by three anonymous reviewers. Funding was provided by a National Science Foundation award (DEB 0543632) to D. Wagner and P. Doak.

## References

Abramoff MD, Magelhaes PJ, Ram SJ (2004) Image processing with ImageJ. Biophotonics Int 11:36–42

Addicott JF, Aho JM, Antolin MF, Padilla DK, Richardson JS, Soluk DA (1987) Ecological neighborhoods—scaling environmental patterns. Oikos 49:340–346

Adjei-Maafo IK, Wilson LT, Thomson NJ, Blood PRB (1983) Effect of pest damage intensity on the growth, maturation, and yield of nectaried and nectariless cotton. Environ Entomol 12:353–358

Barton AM (1986) Spatial variation in the effect of ants on an extrafloral nectary plant. Ecology 67:495–504. doi:10.2307/1938592

Bentley BL (1977) Extrafloral nectaries and protection by pugnacious bodyguards. Annu Rev Ecol Syst 8:407–427. doi:10.1146/annurev.es.08.110177.002203

Bestelmeyer BT (2005) Does desertification diminish biodiversity? Enhancement of ant diversity by shrub invasion in south–western USA. Divers Distrib 11:45–55. doi:10.1111/j.1366-9516.2005.00122.x

Bronstein JL (1998) The contribution of ant–plant protection studies to our understanding of mutualism. Biotropica 30:150–161. doi:10.1111/j.1744-7429.1998.tb00050.x

Canada Natural Resources (2008) Yukon forest health report. Natural Resources, Ottawa

Chamberlain SA, Holland JN (2008) Density-mediated, context-dependent consumer resource interactions between ants and extrafloral nectar plants. Ecology 89:1364–1374. doi:10.1890/07-1139.1

Chamberlain SA, Holland JN (2009) Quantitative synthesis of context dependency in ant–plant protection mutualisms. Ecology 90:2384–2392. doi:10.1890/08-1490.1

Condrashoff SF (1962) A description of the immature stages of *Phyllocnistis populiella* Chambers (Lepidoptera: Gracillariidae). Can Entomol 94:902–909

Condrashoff SF (1964) Bionomics of the aspen leaf miner, *Phyllocnistis populiella* Cham. (Lepidoptera: Gracillariidae). Can Entomol 96:857–874. doi:10.4039/Ent96857-6

Connor EF, Taverner MP (1997) The evolution and adaptive significance of the leaf-mining habit. Oikos 79:6–25

Di Giusto B, Anstett MC, Dounias E, McKey DB (2001) Variation in the effectiveness of biotic defence: the case of an opportunistic ant–plant protection mutualism. Oecologia 129:367–375. doi:10.1007/s004420100734

Doak P, Wagner D, Watson A (2007) Variable extrafloral nectary expression and its consequences in quaking aspen. Can J Bot 85:1–9. doi:10.1139/b06-137

Heads PA, Lawton JH (1985) Bracken, ants and extrafloral nectaries. III. How insect herbivores avoid ant predation. Ecol Entomol 10:29–42. doi:10.1111/j.1365-2311.1985.tb00532.x

Heil M (2008) Indirect defence via tritrophic interactions. New Phytol 178:41–61. doi:10.1111/j.1469-8137.2007.02330.x

Heil M et al (2002) Reduced chemical defence in ant-plants? A critical re-evaluation of a widely accepted hypothesis. Oikos 99:457–468. doi:10.1034/j.1600-0706.2002.11954.x

Heil M, Hilpert A, Kruger R, Linsenmair KE (2004) Competition among visitors to extrafloral nectaries as a source of ecological costs of an indirect defence. J Trop Ecol 20:201–208. doi:10.1017/s026646740300110x

Huntzinger M, Karban R, Young TP, Palmer TM (2004) Relaxation of induced indirect defenses of acacias following exclusion of mammalian herbivores. Ecology 85:609–614. doi:10.1890/03-3056

Kenward MG, Roger JH (1997) Small sample inference for fixed effects from restricted maximum likelihood. Biometrics 53:983–997

Levin SA (1992) The problem of pattern and scale in ecology. Ecology 73:1943–1967. doi:10.2307/1941447

Mondor EB, Addicott JF (2003) Conspicuous extra-floral nectaries are inducible in *Vicia faba*. Ecol Lett 6:495–497. doi:10.1046/j.1461-0248.2003.00457.x

Ness JH (2003) *Catalpa bignonioides* alters extrafloral nectar production after herbivory and attracts ant bodyguards. Oecologia 134:210–218. doi:10.1007/s00442-002-1110-6

Nomikou M, Janssen A, Sabelis MW (2003) Herbivore host plant selection: whitefly learns to avoid host plants that harbour predators of her offspring. Oecologia 136:484–-488. doi:10.1007/s00442-003-1289-1

Östman O, Ives AR (2003) Scale-dependent indirect interactions between two prey species through a shared predator. Oikos 102:505–514. doi:10.1034/j.1600-0706.2003.12422.x

Pemberton RW (1993) Observations of extrafloral nectar feeding by predacious and fungivorous mites. Proc Entomol Soc Wash 95:642–643

Rose USR, Lewis J, Tumlinson JH (2006) Extrafloral nectar from cotton (*Gossypium hirsutum*) as a food source for parasitic wasps. Funct Ecol 20:67–74. doi:10.1111/j.1365-2435.2006.01071.x

Rudgers JA (2004) Enemies of herbivores can shape plant traits: selection in a facultative ant–plant mutualism. Ecology 85:192–205. doi:10.1890/02-0625

Rudgers JA, Strauss SY (2004) A selection mosaic in the facultative mutualism between ants and wild cotton. Proc R Soc Lond B 271:2481–2488. doi:10.1098/rspb.2004.2900

Rudgers JA, Strauss SY, Wendel JF (2004) Trade-offs among anti–herbivore resistance traits: insights from Gossypieae (Malvaceae). Am J Bot 91:871–880. doi:10.3732/ajb.91.6.871

Rutter MT, Rausher MD (2004) Natural selection on extrafloral nectar production in *Chamaecrista fasciculata*: the costs and benefits of a mutualism trait. Evolution 58:2657–2668. doi:10.1554/04-009

Sendoya SF, Freitas AVL, Oliveira PS (2009) Egg-laying butterflies distinguish predaceous ants by sight. Am Nat 174:134–140. doi:10.1086/599302

Shelton AL (2005) Within-plant variation in glucosinolate concentrations of *Raphanus sativus* across multiple scales. J Chem Ecol 31:1711–1732. doi:10.1007/s10886-005-5922-9

Shroff R, Vergara F, Muck A, Svatos A, Gershenzon J (2008) Nonuniform distribution of glucosinolates in *Arabidopsis thaliana* leaves has important consequences for plant defense. Proc Natl Acad Sci USA 105:6196–6201. doi:10.1073/pnas.0711730105

Sugiura S, Abe T, Makino S (2006) Loss of extrafloral nectary on an oceanic island plant and its consequences for herbivory. Am J Bot 93:491–495. doi:10.3732/ajb.93.3.491

Trelease W (1881) The foliar nectar glands of Populus. Bot Gaz 6:284–290. doi:10.1086/325529

US Forest Service (2008) Forest health conditions in Alaska—2007: a forest health protection report. US Forest Service, Alaska Region R10-PR-18

Wagner D, Nicklen EF (2010) Ant nest location, soil nutrients, and nutrient uptake by ant-associated plants: does extrafloral nectar attract ant nests and thereby enhance plant nutrition? J Ecol 98:614–624. doi:10.1111/j.1365-2745.2010.01640.x

Wagner D, DeFoliart L, Doak P, Schneiderheinze J (2008) Impact of epidermal leaf mining by the aspen leaf miner (*Phyllocnistis populiella*) on the growth, physiology, and leaf longevity of quaking aspen. Oecologia 157:259–267. doi:10.1007/s00442-008-1067-1

Wooley SC, Donaldson JR, Gusse AC, Lindroth RL, Stevens MT (2007) Extrafloral nectaries in aspen (*Populus tremuloides*): heritable genetic variation and herbivore-induced expression. Ann Bot 100:1337–1346. doi:10.1093/aob/mcm220

Young BD, Wagner D, Doak P, Clausen TP (2010) Within-plant distribution of phenolic glycosides and extrafloral nectaries in trembling aspen, *Populus tremuloides.* Am J Bot 97:601–610. doi:10.3732/ajb.0900281