Impact of epidermal leaf mining by the aspen leaf miner (*Phyllocnistis populiella*) on the growth, physiology, and leaf longevity of quaking aspen

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Abstract The aspen leaf miner, *Phyllocnistis populiella*, feeds on the contents of epidermal cells on both top (adaxial) and bottom (abaxial) surfaces of quaking aspen leaves, leaving the photosynthetic tissue of the mesophyll intact. This type of feeding is taxonomically restricted to a small subset of leaf mining insects but can cause widespread plant damage during outbreaks. We studied the effect of epidermal mining on aspen growth and physiology during an outbreak of *P. populiella* in the boreal forest of interior Alaska. Experimental reduction of leaf miner density across two sites and 3 years significantly increased annual aspen growth rates relative to naturally mined controls. Leaf mining damage was negatively related to leaf longevity. Leaves with heavy mining damage abscised 4 weeks earlier, on average, than leaves with minimal mining damage. Mining damage to the top and bottom surfaces of leaves had different effects on physiology. Mining on the top surface of the leaf had no significant effect on photosynthesis or conductance and was unrelated to leaf stable C isotope ratio ($\delta^{13}C$). Mining damage to the bottom leaf surface, where stomata are located, had significant negative effects on net photosynthesis and water vapor conductance. Percent bottom mining was positively related to leaf $\delta^{13}C$. Taken together, the data suggest that the primary mechanism for the reduction of photosynthesis by epidermal leaf mining by *P. populiella* is the failure of stomata to open normally on bottom-mined leaves.

Keywords *Populus tremuloides* · *Phyllocnistis populiella* · Herbivory · Leaf mining · Growth

Introduction

Population outbreaks of insect herbivores can have large impacts on the net productivity of forest trees (Mattson and Addy 1975; Schowalter et al. 1986), particularly in the boreal forest where insect outbreaks are common (Bonan and Shugart 1989). Poplars (*Populus* spp.) such as quaking aspen (*P. tremuloides* Michx.) are ecologically and economically important components of boreal forests across North America, subject to recurring outbreaks of several major insect herbivores (Mattson et al. 2001; Coyle et al. 2005). The most common, and best-studied, poplar herbivores feed by chewing, causing heavy defoliation of trees when insect populations are large. Multiple experimental studies indicate that, while poplars can typically survive even high levels of defoliation, large-scale losses of leaf area can decrease growth (Bassman et al. 1982; Bassman and Zwier 1993; Reichenbacker et al. 1996; Kosola et al. 2001) and increase the rate of top dieback (Kosola et al. 2001). Dendroecological approaches confirm that large-scale outbreaks of defoliators such as the forest tent caterpillar (*Macosomas disstria* Hbn) reduce poplar growth (Hogg et al. 2002; Cooke and Roland 2007) and, moreover, may predispose stands to secondary damage by wood-boring insects and fungal pathogens (Churchill et al. 1964). Defoliation appears to have neutral or positive effects on the net photosynthesis of remaining leaves (Bassman and Zwier 1993; Stevens et al.)
Tahiti lime was shown to decrease yield and reduce net photosynthesis (Schaffer et al. 1989). Severe outbreaks of insect herbivores that do not defoliate poplar, such as leaf miners, are less common and their effects less studied, but may also have important impacts on tree growth and physiology (e.g., Raimondo et al. 2003; Nardini et al. 2004). In the boreal forests of North America, quaking aspen is occasionally subject to high population densities of the aspen leaf miner, *Phyllocnistis populiella*. High levels of damage caused by *P. populiella* were recorded throughout northern Canada from the 1940s to the 2000s (Condrashoff 1984; Porter 1976; Natural Resources Canada 2004). In interior Alaska, north of the Alaska Range, high densities of *P. populiella* were noted in 1996 (R. Werner, unpublished data, http://www.lter.uaf.edu). By 2005, over 2,65,000 ha of Alaska sustained obvious leaf miner damage (US Forest Service 2005). *P. populiella* has a strikingly unusual mode of feeding. Whereas most leaf-mining insects consume the photosynthetic cells of the mesophyll (Hering 1951), a small subset of leaf miner taxa including those in the genus *Phyllocnistis* consume only the cells of the epidermis, leaving the cuticle intact (Hering 1951; Condrashoff 1964). While taxonomically rare, outbreaks of epidermal leaf mining insects could have significant ecological impacts through negative effects on plant performance.

Most of our understanding of the physiological effects of leaf mining on plants comes from studies of taxa that consume the leaf mesophyll. Mesophyll mining typically reduces photosynthesis and often stomatal conductance as well (Proctor et al. 1982; Johnson et al. 1983; Trumble et al. 1985; Whittaker 1994). Mesophyll mining damage can also shorten leaf longevity (Pritchard and James 1984). Although epidermal leaf miners do not directly damage the primary photosynthetic cells of the leaf, this form of leaf mining could damage or destroy stomata, thereby reducing photosynthesis and disrupting the water balance (Welter 1989). However, few studies have addressed the effects of epidermal leaf mining on plant performance. Epidermal mining on Tahiti lime was shown to decrease yield and reduce net photosynthesis (Schaffer et al. 1997; Peña et al. 2000). The mechanism behind this effect was, however, not clear.

In this study we investigated the effects of epidermal mining on the performance of naturally occurring aspen trees in interior Alaska during an outbreak of *P. populiella*. We tested the effect of epidermal leaf mining on growth by experimentally reducing leaf miner density over a 3-year period at two sites. We further investigated effects of *P. populiella* mining on leaf longevity and plant physiology. *P. populiella* mines both the top (adaxial) and the bottom (abaxial) surfaces of aspen leaves, but since the stomata are located on the bottom surface only, we expected the effects of mining on the top and bottom leaf surfaces to differ. In particular, we tested the hypothesis that the destruction of guard cells impacts photosynthesis by reducing stomatal conductance. When photosynthesis is chiefly limited indirectly, through decreased stomatal conductance, rather than directly, through damage to photosynthetic tissues, low partial pressure of CO$_2$ in the leaf intercellular spaces should lead to low physiological discrimination against $^{13}$C and a high (less negative) stable C isotope ratio ($\delta^{13}$C) (Farquhar et al. 1982). If mining damage reduces photosynthesis chiefly by limiting CO$_2$ influx, then the extent of mining damage to the bottom leaf surface should relate positively to leaf $\delta^{13}$C.

**Materials and methods**

**Natural history of the herbivore**

*Phyllocnistis populiella* overwinters as an adult and, in interior Alaska, adult moths emerge from diapause in May. Oviposition begins immediately after host tree bud break and continues for about 2 weeks. Eggs are laid on both top and bottom surfaces of young leaves. Eggs sink into the leaf tissue and larvae hatch directly into the epidermal cell layer about 1 week after oviposition. The larvae consume epidermal cell contents as they move, creating an air pocket between the cuticle and the mesophyll that gives mines a silvery-white appearance (Fig. 1). Prognathus mouthparts prevent the larva from tunneling downward or exiting and reentering the leaf, so the larva feeds throughout its development on a single side of a leaf (Hering 1951). During the years covered by this study, it was common for more than one larva to complete development on a single leaf side. After approximately 2-3 weeks of development, the larvae pupate within small leaf folds, usually at the leaf edge. Most larvae have pupated by mid-June. Adults emerge 10-14 days later but do not appear to mate until the following spring.

In interior Alaska during the years of this study, damage to aspen by *P. populiella* was conspicuous and extensive. For example, in a 2006 survey of 90 trees across multiple sites in the Fairbanks area, 99% of aspen trees and 86% of all leaves bore some mining damage; the average leaf was mined over 58% (±41 SD, n = 646) of the top and 46% (±38 SD) of the bottom surface (P. Doak and O. Wagner, unpublished data).

**Growth**

To assess the effects of leaf mining on aspen growth, leaf miner density was experimentally reduced on trees at two study sites during the 2005, 2006, and 2007 growing seasons. Both sites were located in the vicinity of Fairbanks, Alaska: Bonanza Creek Long-Term Ecological Research
and the summit of Ester Dome (ED; 64°52’58”N, 148°04’06”W, elevation 720 m). The density of trees > 20 cm high was 10,850 trees ha−1 at BNZ and 2,345 trees ha−1 at ED. In May of 2005, we haphazardly chose 40 aspen trees <2 m high at each site. Within these natural stands, it was not possible for us to ascertain whether the experimental trees at a site belonged to the same clone. The initial height of experimental trees ranged from 110-186 cm at BNZ and 55-183 cm at ED. Initial diameter was assessed by taking two orthogonal measurements of the trunk at 20-cm height using calipers. Permanent ink was used to draw a ring on the trunk at 20-cm height to ensure that final measurements were made at the same position. Half of the trees at each site were assigned at random to receive insecticide treatments. In May 2005, 2006, and 2007, just following bud break and while leaf miner eggs and young larvae were present, treatment plants were sprayed with the insecticide spinosad (Conserve; Dow AgroSciences, Indianapolis, Ind.; concentration 1.56 ml l−1; applied with a hand-powered pump sprayer to runoff). At BNZ in 2005, and at both sites in 2006, the insecticide was re-applied 3-7 days later. In 2007, the second insecticide application was omitted altogether, in order to reduce the chances of non-target effects on other herbivore taxa. Whenever treatment trees were sprayed with pesticide, control plants were sprayed with an equivalent volume of water.

Leaf damage on experimental trees was assessed between mid-July and mid-August of 2005-2007, after leaf miner larvae had ceased to feed. Herbivory was scored on the seven most proximal (oldest) leaf positions of two shoots per plant in 2005 and three shoots per plant in 2006 and 2007. We visually estimated the percent bottom mining, percent top mining, and the percent of leaf tissue missing and damaged by other herbivores. Our visual estimates of mining damage correlate well with measurements made with image analysis software (Scion Image, Fredrick, Md.; R² > 0.9, Doak and Wagner).

We measured growth of sprayed and control trees in the late summer of 2005-2007. In addition to re-measuring diameter at 20-cm height, we measured the length of the three longest shoots on each plant.

Over the 3 years of the insecticide application experiment, a subset of ramets ceased to produce new leaf tissue above the 20-cm-high ink ring, although some sent out new shoots from the base near the surface of the soil. Top dieback is defined here as the lack of living tissue above 20 cm. Ramets that suffered top dieback were excluded from the trunk diameter data set. All ramets with living shoots were included in the shoot elongation data set, regardless of whether the vegetation occurred above or below the ring.

We analyzed the effect of insecticide application on the dependent variables shoot length (the summed length of the three longest shoots) and change in average trunk diameter using separate repeated measures ANOVAs, with treatment and site as fixed effects, initial stem diameter as a covariate, and year as a within-subjects effect. We analyzed the effect of treatment on the frequency of top dieback for both sites combined using a t-test.

Leaf δ¹³C

We examined the relationship between mining damage and leaf δ¹³C using trees from the growth experiment described above. In July 2005, leaves were collected from each of the 40 experimental trees at one site (ED). We collected three leaves from each of two shoots per tree at leaf positions 1 (oldest, most proximal) or 2, 3 or 4, and 5 or 6. This set of positions covered the range of leaf positions on an average shoot at this site (average from an independent sample = 6.4 leaves/shoot ± 1.7 SD, range 5-15, n=77 shoots). Leaves were placed individually into airtight plastic bags and kept cool while in transit to the laboratory. Leaves were scanned for image analysis to determine area and percent damage, then dried at 60°C for 1 week and reweighed to determine the percentage leaf moisture.

After drying, leaf tissue was ground and sub-samples of 0.8 mg were analyzed for δ¹³C using a Europa GEOS 20-20 continuous flow isotope ratio mass spectrometer. The effects of leaf mining damage on leaf water content and δ¹³C were analyzed using linear mixed models, with insecticide treatment as a fixed effect, percent top mining, percent bottom mining, and leaf position as covariates, and tree as a random effect.
Photosynthesis and conductance

Preliminary work on aspen trees with natural levels of leaf mining damage indicated that photosynthesis was negatively related to the percentage of epidermal leaf mining on the leaf bottom, but unrelated to mining on the leaf top (L. DeFoliart, unpublished data). Preliminary work also established that the extent of mining damage on the top and bottom of leaves was correlated, potentially complicating an interpretation of gas exchange data taken from naturally mined trees. Therefore we tested the effect of leaf mining on gas exchange by experimentally manipulating the presence and absence of leaf miner eggs on a set of aspen trees to produce leaves with damage restricted to one side or the other.

We tested the effect of epidermal leaf mining on photosynthesis and conductance in early June 2006 in a stand of small (<2 m high) aspen on the University of Alaska Fairbanks campus (64°51.462’N, 147°51.342’W, elevation 184 m). Eight trees of unknown genotype were chosen for study because of their proximity to a footpath. On each tree, we haphazardly chose nine shoots. To control for variation in photosynthesis due to leaf position along the shoot (Noormets et al. 2001), we manipulated a single leaf per shoot, located at a constant position (the third leaf from the proximal end of shoot). Leaves were marked with petiole tags and randomly assigned to one of three treatments: mined on the upper surface only, mined on the lower surface only, or mined on neither surface. At the time, all leaves had >1 leaf miner egg on both upper and lower surfaces. Leaf miner eggs were removed from leaves with a metal probe. Care was taken not to pierce the cuticle. Branches with manipulated leaves were immediately bagged. One week later, when leaf miner oviposition had ceased, bags were removed.

Gas exchange parameters were determined in late June after larval feeding was completed over a 2-day period using a portable infrared gas analyzer (LI-6400; LI-COR, Lincoln, Neb.) with an air flow rate of 500 umol m$^{-2}$ s$^{-1}$ and light intensity 750 umol photons m$^{-2}$ s$^{-1}$. The leaf was removed from the chamber, and a digital image of both surfaces was made using a desktop scanner. The percentage mining damage of was determined using image analysis software (Scion Image, Fredrick, Md.).

The effects of leaf mining treatment (none, top only, bottom only) on the dependent variables photosynthesis and conductance were analyzed using linear mixed model ANOVAs, each including tree as a random factor. To determine the relationship between photosynthesis and the extent of mining damage, we regressed photosynthesis against percent mining damage for the top-mined and bottom-mined groups separately.

Leaf longevity

We investigated the effect of epidermal mining on leaf longevity in June 2003, using a set of ten small (<2 m high) trees of unknown genotype located on a south-facing slope near Fairbanks (64°51.27”N 147°51.23”W). For each tree, we haphazardly chose two shoots and marked the petioles of five leaves on each shoot. Within a tree, leaves were paired for position along the shoot. In early June 2003, after oviposition by *P. populiella* had largely ceased, we fumigated one shoot on each tree to kill eggs and young larvae. A commercially available strip impregnated with the pesticide 2,2 dichlorovinyl dimethyl phosphate was loosely wired to the shoot and the shoot and strip were encased in a plastic bag for 90 min. Fumigation was conducted in the early morning under cool temperatures. Following treatment, we observed each leaf weekly until it abscised. The extent of mining on the total leaf surface (damage to the leaf bottom and top combined) was estimated visually each week and categorized and ranked as follows: 0 (no mining), 1 (1-25% of leaf surface mined), 2 (26-50%), 3 (51-75%), and 4 (76-100%).

Leaf longevity was defined as the number of days between the start of the experiment and the last day the leaf was observed attached to the tree. The effect of experimental reduction of leaf mining on leaf longevity was assessed with a paired *t*-test. The relationship between leaf longevity and the last recorded level of mining damage was assessed using Spearman’s rank correlation analysis.

Data analysis-general considerations

Model residuals were examined for normality and homogeneity of variances. Data that violated parametric assumptions and could not be successfully transformed were analyzed using nonparametric tests. All analyses were conducted in JMP IN 5.1 (SAS Institute, Cary, N.C.). Mixed model ANOVAs were analyzed using the restricted residual maximum likelihood method.

Results

Growth

Application of insecticide in 2005, 2006 and 2007 successfully reduced herbivory by the aspen leaf miner on treated trees (Table 1). Although leaf mining represented the majority of leaf damage, chewing, skeletonizing, and galling damage were also observed. Our early season insecticide applications had no statistically significant effect on the percentage of leaf damage caused by non-mining herbivores (Table 1).
In general, trees with experimentally reduced leaf miner damage grew more than controls (Fig. 2). Across 3 years of repeated measurements, insecticide application significantly increased seasonal shoot elongation and diameter expansion at both sites (Table 2). Neither measure of growth changed significantly across years (Table 2). Plants at the higher-elevation site (ED) expanded in diameter at a marginally higher rate than plants at the lower-elevation site, but there was no site-to-site difference in annual shoot elongation (Fig 2, Table 2). Diameter growth was negatively related to initial (2005) diameter, while shoot elongation was unrelated to initial diameter (Table 2).

By 2007, top dieback had occurred on 11% of all insecticide-treated trees and 25% of all controls, a difference that was not statistically significant ($X^2 = 1.4, df = 1, P > 0.05$). Four trees failed to produce any living shoots at all in the third year (one insecticide-treated tree and three controls), a sample that was too small for statistical analysis.

Leaf δ^13C

Leaf δ^13C was positively related to percent bottom mining damage (Fig. 3; $F_{1,178} = 139.9, P < 0.001$) but not significantly related to top mining damage ($F_{1,178} = 3.3, P = 0.07$). Bottom and top mining did not interact ($F_{1,178} = 1.7, P = 0.2$). Leaf position did not influence leaf δ^13C ($F_{1,178} = 1.8, P = 0.2$). Independent of its effect on leaf mining, experimental treatment had no statistically significant effect on leaf δ^13C ($F_{1,36} = 0.3, P = 0.6$).

Photosynthesis and conductance

By selectively removing eggs from leaves, we produced leaves that were completely unmined, mined on the top surface only (mean 78% mined, range 25-100%), or mined on the bottom surface only (mean 69% mined, range 32-95%). Mean net photosynthesis by leaves with bottom mining only was approximately 70% lower than that of top-mined and unmined leaves (Fig 4a; $F_{2,25} = 34.4, P < 0.001$; Tukey-Kramer $P < 0.05$). Photosynthesis by top-mined and unmined leaves did not differ significantly (Tukey-Kramer $P > 0.05$). After controlling for individual differences among trees, photosynthesis was negatively related to percent leaf mining for the bottom-mined leaves ($F_{1,7} = 6.0, P = 0.04$) but unrelated to percent mining on the top-mined leaves ($F_{1,7} = 0.1, P = 0.7$).

Mining damage to the bottom surface of leaves significantly decreased conductance relative to unmined and top-
mined leaves (Fig. 4b; $F_{2,35} = 18.2, P < 0.001$; Tukey-Kramer $P < 0.05$); conductance on unmined and top-mined leaves did not differ significantly ($P > 0.05$). Conductance explained 93% of the variation in photosynthesis (Fig. 4b; $P < 0.001$). Despite the strong influence of conductance, after accounting for its effect on photosynthesis in a regression model, bottom-mined leaves still tended to have significantly more negative residual values, or lower photosynthesis, than either top-mined or unmined leaves (Fig. 4; $F_{2,62} = 12.8, P < 0.001$).

Leaf longevity

Insecticide substantially reduced mining damage on treated shoots in the longevity experiment. The majority of fumigated leaves (45/50) were mined over <25% of the total leaf surface (top and bottom combined), whereas the majority of untreated leaves (45/50) sustained mining damage over >75% of the leaf surface. Leaves with experimentally reduced mining damage remained attached to the plant significantly longer than leaves with natural levels of mining damage (Fig. 5a; paired $t = 14.3, df = 9, P < 0.001$). Across all leaves, the length of time that leaves remained attached to plants was negatively correlated with percent leaf mining damage (Fig. 5b; $r_s = -0.82, n = 100, P < 0.001$). The early loss of heavily mined leaves had no direct impact on *P. populiella*, as moths had eclosed and exited leaves weeks before leaves began to abscise.

Discussion

Epidermal leaf mining by *P. populiella* had a strong negative effect on aspen growth, likely resulting from a combination of lower net photosynthesis and early leaf abscission. Experimental reduction of leaf mining damage increased the average growth of aspen ramets relative to naturally mined ramets across the 3 years of the study. Relative to sprayed plants with $<10\%$ mining damage, natural mining damage to about 30% of the bottom surface, the average in most years, reduced shoot length by 30 and 25%, and seasonal diameter growth by 25 and 50%, at our two study sites, respectively.

Although *P. populiella* does not consume the photosynthetic tissues of the mesophyll, damage to the epidermis resulted in clear impacts on aspen photosynthesis. However, mining damage to the two surfaces of aspen leaves had very different effects on plant physiology. Photosynthetic rate was unaffected by the mining damage most conspicuous to observers: that on the top of aspen leaves. In contrast, the less noticeable bottom mining substantially reduced the photosynthetic rate. Both gas exchange and

Table 2 Multivariate, repeated measures ANOVA testing the effect of insecticide treatment on two, log-transformed measures of plant growth at two sites. Significant ($<0.05$) $P$-values are presented in bold.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>df</th>
<th>F</th>
<th>P</th>
<th>df</th>
<th>F</th>
<th>P</th>
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</thead>
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<tr>
<td>Insecticide (I)</td>
<td>1.70</td>
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<td>0.008</td>
<td>1.63</td>
<td>14.3</td>
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<tr>
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<td>0.5</td>
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<td>Initial stem width (W)</td>
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<td>1.70</td>
<td>0.2</td>
<td>1.63</td>
<td>25.7</td>
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Within subjects

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<th>Wilks’ $\lambda$</th>
<th>P</th>
<th>df</th>
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Fig. 3 Relationship between mining damage to the leaf bottom and leaf $\delta^{13}C$.
foliar C isotopic measurements were consistent with the hypothesis that mining damage to the bottom side of the leaf prevents normal opening of the stomata. Mining damage to the leaf bottom, where the stomata are located, caused a significant reduction in water vapor conductance, while mining damage to the top did not. Moreover, a positive relationship between leaf $^{13}$C and percent bottom mining indicated that bottom mining reduced the partial pressure of CO$_2$ in the intercellular spaces of the leaf during photosynthesis (Farquhar et al. 1989). Likely, the contents of guard cells are consumed by leaf miners along with other epidermal cells, leaving the stomata unable to open properly. An analogy might be found in the effects of damage by the apple rust mite (Acclus schlechtendalli), which feeds exclusively by puncturing the epidermal cells of apple leaves. Mite-damaged guard cells were found to be desiccated, partially closed, and unresponsive, leading to a negative correlation between mite damage and CO$_2$ uptake by apple leaves (Spieser et al. 1998).

The effect of herbivory on photosynthesis can extend well beyond portions of the leaf that suffer direct damage (Zangerl et al. 2002.). To a large extent, the leaf mining-related decrease in photosynthesis we report here was consistent with changes to stomatal conductance. However, after controlling for conductance, photosynthesis of bottom-mined leaves was still somewhat lower than that of unmined and top-mined leaves (Fig. 4b), suggesting that the effects of mining damage to the lower leaf epidermis extend to other, yet unidentified, aspects of leaf physiology.

On average, leaves with 50% of the epidermal surface mined abscised more than 2 weeks earlier, and leaves with
100% mining more than 4 weeks earlier, than undamaged leaves. Mesophyll leaf mining has been reported to accelerate leaf loss in several plant taxa as well (Faeth et al. 1981; Pritchard and James 1984). Early leaf loss by the European horse chestnut (Aesculus hippocastanum) in response to infestation by the palisade-mining moth Cameraria oridella is estimated to cause about 30% loss of net primary productivity (Nardini et al. 2004). The impact of early leaf loss on aspen growth may be particularly significant at high latitude, where the growing season is already short.

We cannot dismiss the possibility that the insecticide treatment we used to reduce leaf miner densities contributed to the higher growth rate of sprayed plants, through effects on physiology. However, in general, insecticides have a neutral or negative effect on photosynthesis (Murthy 1983; Abdel-Reheem et al. 1991; Youngman et al. 1990; Krugh and Miles 1996). There is no evidence that spinosad, the pesticide used in our growth study, Significantly affects gas exchange or yield relative to untreated controls (Haile et al. 1999,2000). In one study, a transient increase in photosynthesis by alfalfa was noted in a small subset of plots shortly after spinosad application, but this effect was neither consistent nor repeatable (Haile et al. 1999). High growth rates of sprayed plants also do not appear to reflect non-target effects of pesticide on other insect taxa, as leaf damage by other herbivores was low overall and not significantly affected by treatment.

We conclude that herbivory by the epidermal leaf miner P. populiella causes previously unappreciated impacts on the performance of quaking aspen. Leaf mining damage to the bottom of the leaf was particularly harmful, leading to a reduction in photosynthesis. Sustained, large population sizes of the leaf miner P. populiella in the boreal forests of North America may be expected to have considerable effects on the productivity of aspen, an ecologically and economically important tree species.

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