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Research paper

The leaf miner *Phyllocnistis populiella* negatively impacts water relations in aspen

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Within the North American boreal forest, a widespread outbreak of the epidermal leaf miner *Phyllocnistis populiella* Cham. has damaged quaking aspen (*Populus tremuloides* Michx.) for nearly 20 years. In a series of experiments, we tested the effects of feeding damage by *P. populiella* on leaf water relations and gas exchange. Relative to insecticide-treated trees, the leaves of naturally mined trees had lower photosynthesis, stomatal conductance to water vapor, transpiration, water-use efficiency, predawn water potential and water content, as well as more enriched foliar δ^{13}C. The magnitude of the difference between naturally mined and insecticide-treated trees did not change significantly throughout the growing season, suggesting that the effect is not caused by accumulation of incidental damage to mined portions of the epidermis over time. The contributions of mining-related stomatal malfunction and cuticular transpiration to these overall effects were investigated by restricting mining damage to stomatous abaxial and astomatous adaxial leaf surfaces. Mining of the abaxial epidermis decreased photosynthesis and enriched leaf δ^{13}C, while increasing leaf water potential and water content relative to unmined leaves, effects consistent with stomatal closure due to disfunction of mined guard cells. Mining of the adaxial epidermis also reduced photosynthesis but had different effects on water relations, reducing midday leaf water potential and water content relative to unmined leaves, and did not affect δ^{13}C. In the laboratory, extent of mining damage to the adaxial surface was positively related to the rate of water loss by leaves treated to prevent water loss through stomata. We conclude that overall, despite water savings due to closure of mined stomata, natural levels of damage by *P. populiella* negatively impact water relations due to increased cuticular permeability to water vapor across the mined portions of the epidermis. Leaf mining by *P. populiella* could exacerbate the negative effects of climate warming and water deficit in interior Alaska.

Keywords: epidermal leaf mining, gas exchange, herbivory, insect outbreak, water balance.

Introduction

Quaking aspen (*Populus tremuloides* Michx.) has sustained growth declines and dieback associated with a combination of insect infestation and drought within the northwest portion of its range (Chen et al. 2018, Trugman et al. 2018, Cortini and Comeau 2019). In Alaska and Yukon, where average temperatures have increased more rapidly than the global average (IPCC 2013), a widespread and persistent outbreak of the aspen leaf miner *Phyllocnistis populiella* Cham. has damaged aspen for nearly two decades (USDA Forest Service 2002, 2019, Reich et al. 2013, Yukon Energy, Mines and Resources 2019). Studies employing a variety of approaches, including experimental insect suppression, retrospective analysis of tree rings and remote sensing of productivity, indicate that infestation by *P. populiella* negatively impacts ramet growth and persistence (Wagner et al. 2008, Wagner and Doak 2013, Cahoon et al.

2018, Boyd et al. 2019). Growth by aspen was strongly and negatively related to midsummer vapor pressure deficit and insect-related mortality over a period of time that coincides with the *P. populiella* outbreak (Trugman et al. 2018), suggesting that feeding damage by *P. populiella* may interact with vapor pressure deficit at the leaf surface (Dang et al. 1997) to affect aspen physiology. Leaf mining by *P. populiella* is known to reduce photosynthesis (Wagner et al. 2008) and because it damages the epidermis, the leaf tissue primarily responsible for water regulation, impacts on water relations are also likely.

Phyllocnistis populiella larvae feed solely within the leaf epidermis by puncturing cells and consuming the fluid cell contents (Condrashoff 1964). Larval feeding disengages the superficial cell membrane, cell wall and cuticle from the deeper, living tissues of the leaf, forming an air-filled space that appears as a pale serpentine track (Figure 1). Stomata occur only on the abaxial surface of aspen leaves (Rushin and Anderson 1981 and confirmed for our study sites), and when larvae feed on the abaxial surfaces of aspen leaves they kill guard cells. Mining damage to the abaxial surface is negatively related to stomatal conductance and photosynthesis and positively related to leaf δ^{13}C, suggesting that stomata in mined regions are non-functional and closed (Wagner et al. 2008). Leaves with closed stomata due to mining might be expected to retain more water than unmined leaves. However, epidermal mining could have a second and opposing effect on water relations if mining compromises the water-holding properties of the cuticle, due to changes in the properties of the cuticle caused by drying or to fissures caused by incidental damage to the mined epidermis. For example, breaks in the cuticle of citrus leaves after feeding by the epidermal miner *P. citrella* led to increased transpiration coupled with decreased photosynthesis (Raimondo et al. 2013). Although plants are capable of healing small breaches in the cuticle through self-assembly of the epicuticular waxes (Koch et al. 2009), other mechanisms by which living plants heal wounds to the epidermis (e.g., Dean and Kolattukudy 1976, Skene 1981) would not be expected to function once the epidermis was disassociated from living tissue. Negative effects associated with increases in cuticular permeability might be expected to increase throughout the growing season, due to an accumulation of incidental damage to mined portions of epidermis.

The goal of this study was to test the effects of epidermal leaf mining on water relations of aspen trees during a *P. populiella* outbreak. To meet this goal, we conducted a series of experiments. First, in order to quantify the overall effects of *P. populiella* feeding damage, we compared gas exchange, predawn and midday water potential, water content and δ^{13}C by free-living trees sustaining ambient levels of insect damage versus those on which the leaf miner density was experimentally reduced. We tested whether negative impacts to water balance increase over time by measuring plants repeatedly throughout a growing season. Second, in order to disentangle the effects of stomatal closure and cuticular permeability on water relations, we conducted another field experiment in which we restricted the location of leaf mining damage to either the astomatous adaxial or stomatous abaxial leaf surface and compared gas exchange, water potential, water content and δ^{13}C to leaves with no damage. Our hypothesis was that mining-related effects on the permeability of the epidermis would dominate on adaxially mined leaves, while stomatal closure would dominate effects on abaxially mined leaves. Third, we further investigated the effects of adaxial mining damage on cuticular permeability by testing in the laboratory whether the percentage mining damage to the adaxial surface is related to the rate of water loss in leaves with the abaxial surface sealed to prevent water loss through stomata.

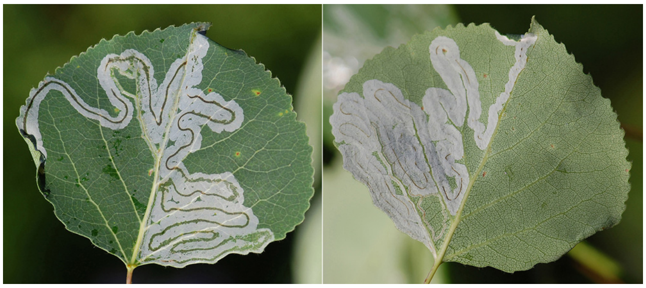

Figure 1. Serpentine mines caused by *P. populiella* to the adaxial (left) and abaxial (right) surfaces of aspen leaves.

Materials and methods

Effects of leaf mining on gas exchange and leaf water relations over time

This portion of the study was conducted at two sites, ‘BNZ’ (64.7094°, −148.3269°) and ‘P327’ (64.7157°, −148.5842°), located 12.3 km apart, 30–40 km west of Fairbanks, AK, during 2016 and 2017 (Table 1). Both sites were characterized by southern exposure and a moderate density of aspen (Table 1). At each site we established a plot ~1600 m^2 in area, and within each plot we haphazardly chose 20 seedling and sapling aspen (n = 40 total) ranging in height from 1.0 to 4.3 m (mean = 2.5 m, SD = 0.8). Because the genetic identity of plants within sites was unknown, we apply the term ‘ramet’ to these individuals hereafter. Half of the focal ramets were randomly assigned to insecticide treatment and treated twice in early June of both 2016 and 2017 with the insecticide spinosad (Conserve SC, EPA registration number 62719-291; Dow AgroSciences, Indianapolis, IN, USA, applied to runoff at a concentration of 1.56 ml l^{-}). Insecticide was applied to individual plants with a hand sprayer during periods of low air movement, with care not to spray neighboring trees. We chose spinosad because studies indicate it does not affect

Table 1. Characteristics of study sites used in 2016 and 2017 to compare leaf physiological variables of ramets subject to natural levels of leaf mining with those treated with insecticide. Data are means with standard deviations in parentheses where appropriate. BNZ stands for Bonanza Creek long-term ecological research site; P327 stands for mile marker 327 on the parks highway (highway 3). Both are near Fairbanks, AK.

Characteristic	Site BNZ	Site P327
Latitude and longitude	64.7094°, −148.3269°	64.7157°, −148.5842°
Canopy cover (%)	23 (13)	52 (14)
Slope (%)	1.1 (1.4)	20.5 (0.2)
Aspect (degrees)	151	173
Density aspen trees (n ha^{-1})	0	0
Density aspen saplings (n ha^{-1})	4 250	10,000
Density aspen seedlings (n ha^{-1})	375	3 333
Total basal area of trees (m^2 ha^{-1})	4.7	8.7
Total basal area aspen (m^2 ha^{-1})	1.3	1.9
Other tree species	*Betula neoalaskana* *Populus balsamifera*	*Betula neoalaskana* *Picea glauca*

gas exchange (Haile et al. 1998, 2000, Spiers et al. 2006) and because, when applied early in the season, it is effective against *P. populiella* leaf miners but relatively ineffective against externally feeding herbivores (e.g., Wagner and Doak 2013). The other half of the focal ramets at each site were sprayed with an equivalent volume of water and served as naturally damaged controls. In 2017, we replaced a total of nine ramets (six at BNZ and three at P327) that were excessively damaged by browsing over winter with new aspen of similar size located nearby.

Estimates of herbivory were made two ways. First, we conducted a single, non-destructive survey of overall leaf damage on each ramet in July of each year, after mining damage had stabilized. On each ramet, a single observer estimated visually the percentage of leaf area mined and missing for the seven leaves proximal to the main stem on each of three haphazardly selected shoots. Leaf damage was averaged within ramets and the effect of treatment and site on the average percentage of leaf area mined and missing analyzed using general linear models. Second, a comparison of changes in leaf damage over time was made using image analysis of leaves harvested following physiological measurements throughout the season. Both sides of each leaf were scanned while fresh using a standard desktop scanner. Total leaf area, area mined on both leaf surfaces and area missing were quantified using ImageJ software (ImageJ, National Institutes of Health, Bethesda, MD, USA). Damage was calculated as a percentage of leaf surface area. Estimates of leaf mining damage using visual estimation and image analysis by our research team are highly related ($R^2 > 0.9$).

Measurements of gas exchange and water potential were made on six occasions during the 2016 growing season, approximately every 2 weeks from May through August; dates of measurement and environmental conditions are in Table S1a available as Supplementary Data at *Tree Physiology* online. For each type of measurement (gas exchange, midday water potential and predawn water potential), we sampled a single, unique leaf occupying the third to fifth position from the proximal end of the shoot. Gas exchange was measured between 12:00 and 14:30h while leaves were attached to the plant using a portable infrared gas analyzer (LI-6400; LI-COR, Lincoln, NE, USA). The cuvette was maintained at a photosynthetically active radiation of 1500 μmol m^{-2} s^{-1} using an LED blue/red light source (Li-Cor 6400-02B), CO_2 concentration was set to 400 μmol mol^{-1} air, and flow was set to 500 $μmol^{-1}$. Net CO_2 assimilation rate (A), stomatal conductance for water vapor (g_s) and transpiration (Tr) were calculated using the LI-6400 analysis program, based on von Caemmerer and Farquhar (1981). Intrinsic water-use efficiency (WUE) was calculated as the ratio of A to Tr. Leaf water potential (Ψ) was measured predawn between 02:00 and 04:30H (Ψ_{PD}) and after midday between 13:00 and 15:00H (Ψ_L) using a portable pressure chamber (PMS Instrument Co., Albany, OR, USA; Model 600). The pressure chamber was set up near the focal plants and, to avoid water loss, each leaf was cut at the petiole immediately before the measurement was made. The leaf was sealed into the chamber with the petiole protruding, and the blade subjected to increasing pressure until fluid became visible under magnification at the cut end of the petiole. After measurement, each leaf was placed in a plastic bag within a cooler and returned to the laboratory for quantification of leaf damage.

When analyzing these data, we aimed to test both whether there was a main effect of treatment and whether the magnitude of the treatment effect increased over time, as would be indicated by a significant interaction between treatment and time. Dependent variables included A, g_s, Tr, WUE, Ψ_L and Ψ_{PD}. Leaf mining damage was still accumulating in May when we initiated measurements. To avoid confounding the effects of increasing insect damage with the effect of wear and tear on the mined cuticle, we separated the analysis of data from the first measurement (27 May 2016) from the measurements made later, after leaf mining damage had stabilized. Data from the 27 May date of measurement were modeled as the fixed effect of treatment and the random effects of ramet and site.

Data from the rest of the measurement dates were used to test the main hypotheses. Mean physiological measurements varied with environmental conditions on the day of measurement (see Table S1b available as Supplementary Data at *Tree Physiology* online), and these variations were not a focus of the study. We therefore standardized each dependent variable across sampling dates by calculating the difference between each data point and the overall mean for all ramets at each combination of site and sampling date. We modeled the standardized data for each dependent variable separately as the fixed effects of treatment and the interaction between treatment and time (with time coded as a continuous variable), and the random effect of individual ramet.

We measured water content and carbon isotopic composition (δ^{13}C) of leaves in 2017. Two leaves were collected from each of the 40 ramets on two dates early and late in the growing season: 30 June and 14 August. Leaves were placed immediately into plastic bags and stored in a cooler for transport to the lab. Following removal of the petiole, leaves were weighed to the nearest 0.0001 g, scanned on both sides with a desktop scanner, dried at 60 °C, and reweighed. Water content was calculated as the percentage of fresh mass composed of water. Leaf δ^{13}C was measured using an isotope ratio mass spectrometer (Finnigan MAT Delta Plus, San Jose, CA, USA). Water content and carbon isotopic composition were modeled as the fixed effects of treatment and time (coded as a nominal variable), and the random effects site and individual ramet.

Respective effects of abaxial and adaxial leaf mining

To better understand the mechanism by which epidermal leaf mining acts on leaf physiology, we restricted leaf mining to one or the other side of leaves and compared measurements of water potential, gas exchange, δ^{13}C and water content to leaves with no mining damage. In May of 2018, we haphazardly selected 14 aspen saplings (average 202 cm tall, SD = 50) within a single, 900 m^2 site with southern exposure on the University of Alaska Fairbanks campus (64°51′31″ N, 147°51′24″ W, elevation 190 m). On each of 10 shoots per ramet, we removed *P. populiella* eggs from three leaves to produce one leaf with adaxial-only mining, one leaf with abaxial-only mining and one leaf with no mining. Treatments were randomly applied with respect to leaf position along the shoot. We surveyed leaves during the following weeks to remove any new eggs laid on treated leaf surfaces and discarded shoots damaged by other types of herbivores. The application of treatments within shoots and ramets allowed us to fully discount any possible effect of environmental variation (e.g., soil moisture or atmospheric conditions) on the resulting leaf physiology.

Measurements of Ψ and gas exchange were made on 13 and 31 July using methods described previously. On each day of measurement, we first measured Ψ_{PD} of all three treated leaves from each of two shoots per ramet. After measurement, leaves were immediately sealed in plastic bags, placed in a cooler, returned to the laboratory, weighed fresh without the petiole and then refrigerated. During the afternoon of the same day, gas exchange and Ψ_{L} were measured on all three treated leaves of an additional two shoots per ramet. Gas exchange measurements were made while leaves were attached to plants, then leaves were snipped at the petiole and Ψ_{L} measured. Immediately after measurement, all leaves were bagged and kept cool for transport to the laboratory, where they were weighed fresh, scanned for image analysis of damage, dried and reweighed. Water content and herbivore damage were determined for all 336 leaves, and leaf δ^{13}C measured on a subset of 84 leaves (28 per treatment), using the methodology described previously.

The dependent variables were modeled as a function of the fixed effect of treatment and the random effects of ramet and shoot nested within ramet. In this experiment, the two dates of measurement were only 2 weeks apart and were intended as a means of increasing the sample size and generalizability rather than a test of change over time, and therefore date was included as a random, rather than a fixed, effect. Following a significant treatment effect, means were compared using Tukey-Kramer HSD tests.

Relationship between leaf mining and water loss in the laboratory

A third experiment tested whether the rate of leaf water loss was positively related to leaf mining damage on the astomatous adaxial surface. Briefly, we sealed the stomatous abaxial surface with wax and subjected leaves to a short period at very low humidity, following Van Gardingen and Grace (1992). On the evening before each of two trials, conducted in July and August 2017, a single stem was collected from each of 30–40 haphazardly selected aspen ramets (0.5–3 m tall) at two sites (site 'WR', 64. 8587°, −147.8561° and site 'RP', 64.8177°, −147.9735). Stems were transported to the laboratory, where they were recut underwater and stored overnight in the dark within a sealed container to promote full hydration. In the morning, one leaf with 0% mining on the abaxial surface and 0–100% mining on the adaxial surface was selected from each ramet sampled. Each leaf was weighed, after which the abaxial surface and cut surface of the petiole were coated with a synthetic polyester wax with a low (37 °C) melting point (Electron Microscopy Sciences, Hatfield, PA, USA). Several minutes later, when the wax had solidified, each leaf was reweighed and placed into an airtight chamber onto a wire mesh surface suspended 2–3 cm above a layer of desiccant crystals. The environment within the chamber was monitored every minute with a small data logger; across trials, the chamber averaged 22.0 °C (SD = 0.2, *n* = 400) and relative humidity 3.9% (SD = 7.6). After 200 min, leaves were removed and reweighed. Wax was removed by rinsing under warm water.

Leaves were scanned for image analysis of area and damage, dried and reweighed to determine dry mass and initial water content.

We assumed leaves were fully hydrated at the start of the experiment and calculated leaf relative water content (RWC) as follows:

$$\mathrm{RWC} = \frac{F_{200} - D}{F_0 - D} \bullet 100$$

where F_0 is the initial fresh mass of waxed leaves, F_{200} is the final fresh mass of waxed leaves at 200 min and D is the leaf dry mass. The influence of leaf mining on RWC was analyzed using a general linear model with percentage adaxial mining, leaf area, the interaction between leaf mining and area, and site as explanatory variables. Trial was included in a preliminary model but dropped due to lack of significance ($P = 0.78$).

Results

Effects of leaf mining on gas exchange and leaf water relations over time

Ambient levels of leaf mining damage by *P. populiella* were high across both years of the study (Figure 2a). Leaf mining affected 68% (SD = 32, $n = 40$) and 52% (SD = 33) of the adaxial and abaxial leaf surfaces, respectively, in 2016 and 52% (SD = 20) and 32% (SD = 15) of adaxial and abaxial surfaces in 2017. Treatment of aspen ramets with insecticide resulted in a 4.6-fold reduction in the average percentage of leaf surface mined in 2016 ($F_{1,36} = 50.88$, $P < 0.0001$) and a 10.4-fold reduction in 2017 (Figure 2a; $F_{1,30} = 84.91$, $P < 0.0001$). In contrast to leaf mining, the percentage of leaf area removed by ectophagous herbivores was much lower overall and did not respond to treatment in either year (Figure 2b; $P > 0.52$). On naturally damaged (control) ramets in 2016, the extent of leaf mining by *P. populiella* was relatively low in late May when physiological measurements began, then increased and stabilized within 2 weeks (Figure 3; $F_{1,354} > 9.00$, $P < 0.0001$). Mining damage to the adaxial surface exceeded damage to the abaxial surface on all dates following May sampling date (Figure 3; $t > 2.5$, DF = 59, $P < 0.02$).

During the first set of physiological measurements in late May, while leaf damage was still relatively low, treatment effects on gas exchange and leaf water potential were modest or non-existent (Figure 4a–f, left of dashed line). There were no statistically significant effects of insecticide treatment on A, g_s, Tr, WUE or Ψ_{PD} ($P > 0.085$), but Ψ_L was somewhat more negative for insecticide-treated ramets than naturally damaged controls (Figure 4e; $F_{1,35} = 4.38$, $P = 0.044$).

After leaf mining damage stabilized, insecticide treatment affected the mean of most physiological processes measured throughout the rest of the growing season. Relative to naturally mined ramets, those treated with insecticide had significantly higher mean levels of A, g_s, Tr and WUE (Figure 4a–d and g–i; Table 2). Leaf Ψ_L was somewhat more negative for insecticide-treated ramets than controls (Figure 4e and j), although this difference was not statistically significant (Table 2; $P = 0.051$).

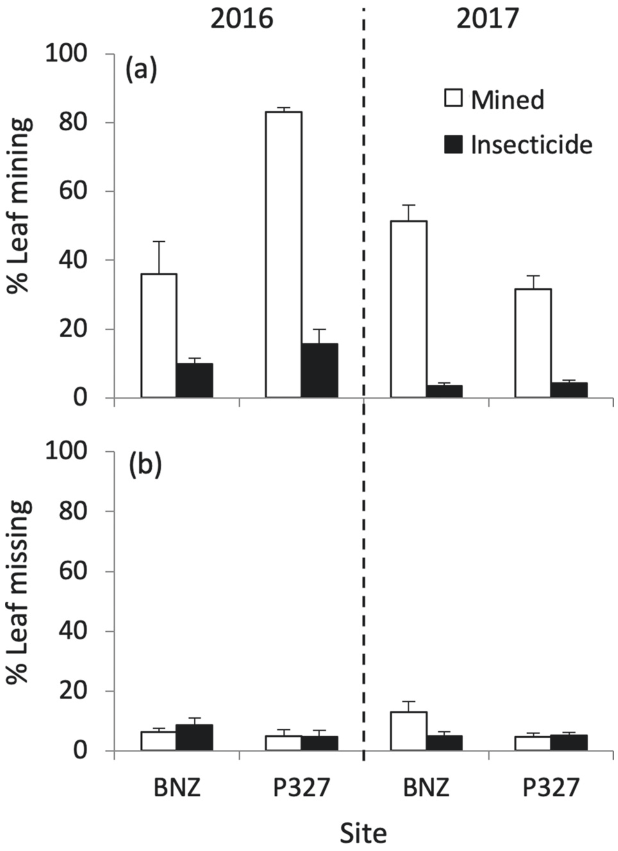

Figure 2. Average leaf damage of untreated trees (controls) and trees treated with insecticide across 2016 and 2017. (a) Percentage of the total leaf surface mined by *P. populiella*. (b) Percentage of leaf area removed by externally feeding herbivores. $N = 40$ trees.

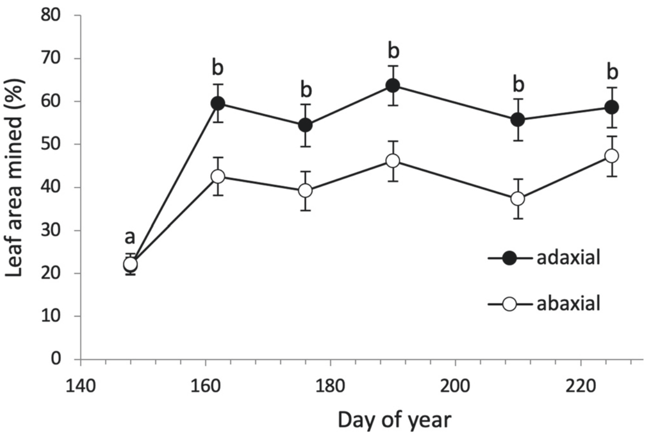

Figure 3. Development of leaf mining damage to the top and bottom surfaces of leaves for unmanipulated (control) aspen plants across six dates of measurement in 2016. $N = 10$ leaves per treatment and day. Different lowercase letters indicate significant (Tukey HSD, $P < 0.05$) differences in leaf mining across time and apply to both upper and lower leaf surfaces.

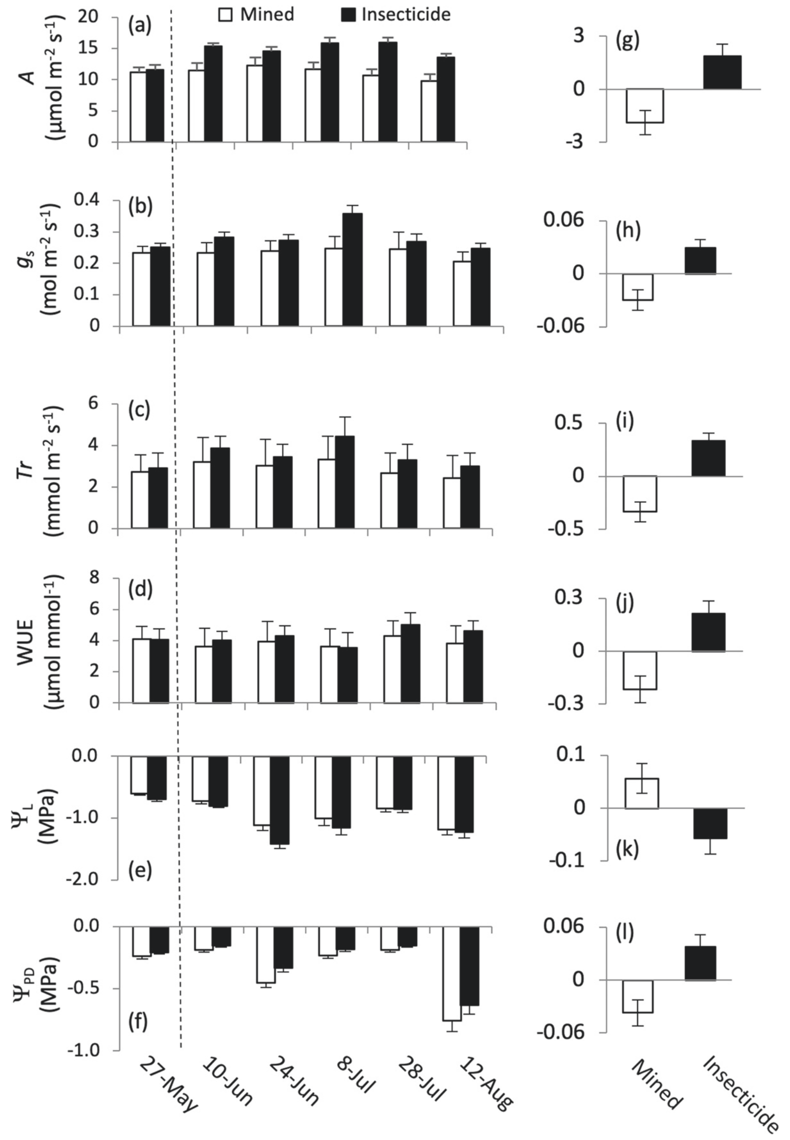

Figure 4. Effect of insecticide treatment on photosynthesis and water relations by 40 aspen seedlings and saplings at two sites measured repeatedly during 2016: net photosynthesis (A), stomatal conductance (g_s), transpiration (Tr), water-use efficiency (WUE), leaf water potential measured at midday (Ψ_L) and leaf water potential measured predawn (Ψ_{PD}). Panels (a) through (f) illustrate mean values at each of the six dates of measurement. Filled bars indicate insecticide-treated trees; open bars indicate unsprayed controls. The dashed line indicates the inter-measurement period during which leaf mining damage stabilized. Panels (g) through (l) illustrate the mean values for insecticide-treated and control plants post-27 May after the data were standardized for site and date of measurement. For all graphs, error bars represent mean standard errors.

During predawn hours, the overall mean Ψ_{PD} was 3-fold less negative than Ψ_L, likely reflecting the combination of nighttime closure of stomata, cooler temperatures and higher humidity (environmental means, see Table S1a available as Supplementary Data at *Tree Physiology* online). Treatment effects on Ψ before dawn were opposite to the trend at midday: insecticide treatment increased mean Ψ_{PD} relative to controls (Figure 4f and k; Table 2). In no case was there a significant interaction between treatment and time (Table 2).

Experimental reduction of leaf mining damage had a positive effect on leaf moisture content. Mean leaf water content of insecticide-treated ramets (overall mean 54.7%, SE = 0.6, n = 85) was significantly greater than that of the control ramets (50.5%, SE = 0.7, n = 91) (Figure 5a; $F_{1,38}$ = 11.00, P = 0.002). Leaf water content was higher in June than in August (Figure 5a; $F_{1,138}$ = 140.54, P < 0.0001). Because leaf moisture was measured only twice during the year, it is not clear whether the difference between months represents a long-term decline in moisture across the season or a short-term response to environmental conditions. However, it is clear that the magnitude of the treatment effect did not vary between June and August (Figure 5a; $F_{1,138}$ = 0.00, P = 0.99), again suggesting that the negative effects of leaf mining on water retention did not intensify through the season.

Table 2. Statistical analysis of water potential and gas exchange data from insecticide-treated (I) and control (C) aspen across five measurement dates during the 2016 growing season. Data were standardized prior to analysis; see text for details. See Figure 4 for means and standard errors; *N* = 40 trees.

Dependent variable	Source of variation	*F*	DF	*P*	Trend
A	Treatment	13.31	1,37	**0.0008**	I > C
	Treatment ∗ Date	0.17	1,158	0.68	
g_s	Treatment	4.21	1,37	**0.047**	I > C
	Treatment ∗ Date	0.16	1,156	0.69	
Tr	Treatment	8.81	1,37	**0.005**	I > C
	Treatment ∗ Date	0.18	1,156	0.67	
WUE	Treatment	36.78	1,37	**0.002**	I > C
	Treatment ∗ Date	2.87	1,158	0.098	
$\boldsymbol{\Psi}_L$	Treatment	3.18	1,38	0.051	I < C
	Treatment ∗ Date	0.92	1,162	0.34	
$\boldsymbol{\Psi}_{PD}$	Treatment	11.06	1,37	**0.002**	I > C
	Treatment ∗ Date	0.73	1,159	0.39	

P-values significant at < 0.05 appear in bold font

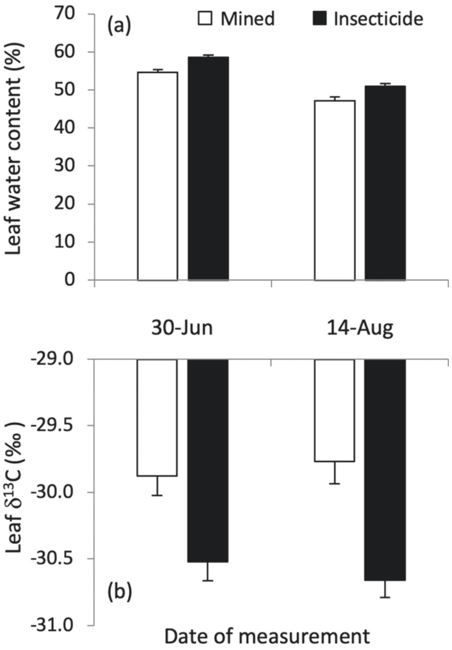

Figure 5. The effect of experimental reduction of leaf mining damage on (a) leaf water content and (b) leaf δ^{13}C of aspen plants (*n* = 40 plants; error bars are mean standard errors).

On average, the δ^{13}C of insecticide-treated leaves was 0.72% more depleted than that of the more heavily mined controls (Figure 5b; $F_{1,36} = 8.8$, $P = 0.005$). There was no statistically significant change in leaf isotopic composition between June and August ($F_{1,118} = 1.26$, $P = 0.26$), and no change in the magnitude of the treatment effect across time (Figure 5b; $F_{1,118} = 0.68$, $P = 0.41$).

Effects of abaxial and adaxial leaf mining

Selective removal of *P. populiella* eggs effectively produced leaves with mining damage to only the adaxial surface (68% mining on average), to only the abaxial surface (50% mining on average), or absent from both surfaces. Mining damage to surfaces from which eggs were experimentally removed affected ≤1% of the surface (see Table S2 available as Supplementary Data at *Tree Physiology* online). Both adaxial and abaxial mining damage decreased *A* relative to leaves with no mining, although the negative effect of abaxial mining exceeded that of adaxial mining (Figure 6a). Adaxial mining had no effect on g_s or *Tr*, whereas abaxial mining decreased both relative to the unmined and adaxial-mined conditions (Figure 6b and c). Mining damage to both leaf surfaces decreased WUE relative to unmined leaves, but for different reasons: adaxial mining decreased *A* (by 13% relative to unmined leaves) without affecting *Tr*, whereas abaxial mining decreased both *A* and *Tr* but affected *A* more strongly (by 25% relative to unmined) than *Tr* (by 17% relative to unmined; Figure 6d). Adaxial and abaxial mining damage had opposite effects on Ψ_L: adaxial mining significantly decreased Ψ_L relative to unmined leaves, whereas abaxial mining, consistent with its negative effect on *Tr*, significantly increased Ψ_L (Figure 6e). During predawn hours, there was no significant difference in Ψ_{PD} among treatments (Figure 6f).

Adaxial and abaxial mining differed in their effects on the more integrative measures of leaf physiology: leaf water content and δ^{13}C. Mining of the adaxial surface significantly reduced leaf water content, whereas mining of the abaxial surface significantly increased water content relative to unmined leaves (Figure 6g). Adaxial mining did not alter leaf δ^{13}C, whereas abaxial mining significantly enriched δ^{13}C relative to unmined leaves (Figure 6h).

Relationship between leaf mining and water loss in the laboratory

The RWC of leaves declined as mining damage to the adaxial leaf surface increased (Figure 7; $F_{1,62} = 10.10$, $P = 0.0023$, $\beta = -0.06$). Leaves from different sites differed in RWC ($F_{1,62} = 9.01$, $P = 0.039$) but responded similarly to variation

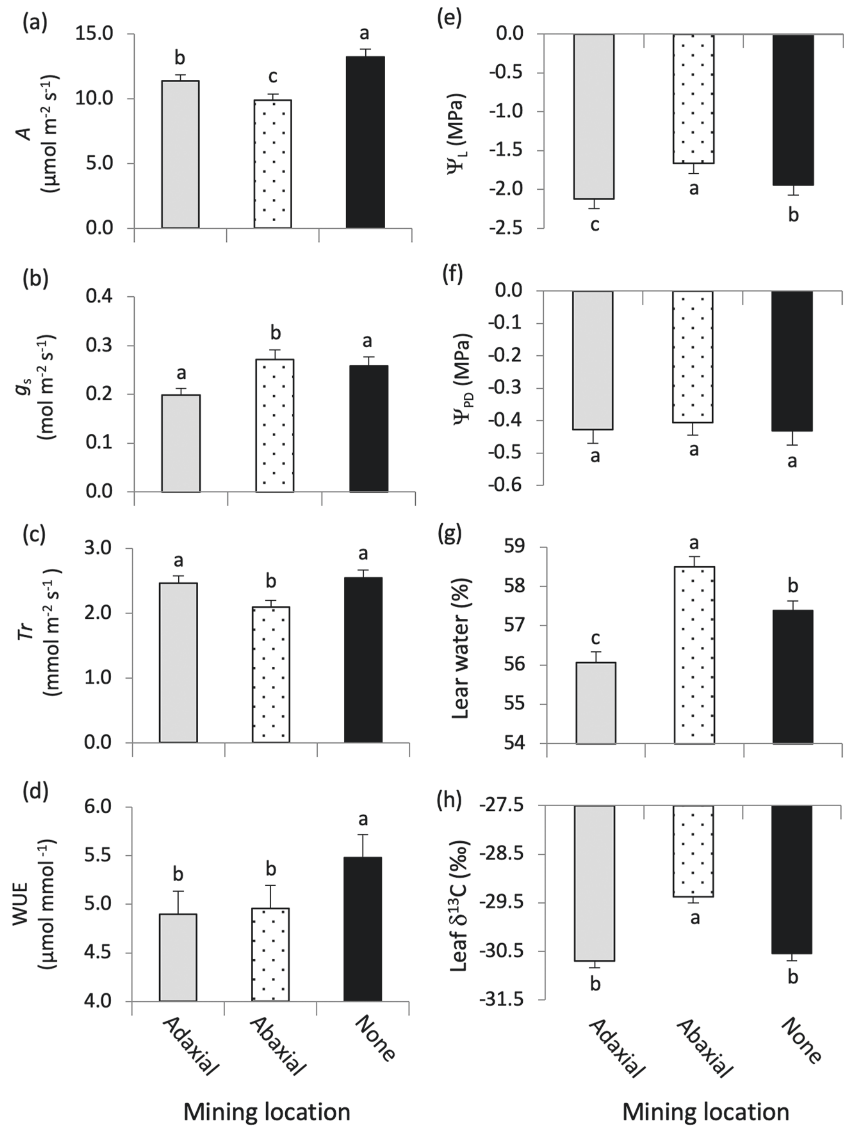

Figure 6. Physiological responses of leaves on which mining damage was either restricted to one side or prevented altogether: net photosynthesis (A), stomatal conductance (g_s), transpiration (Tr), water-use efficiency (WUE), leaf water potential measured at midday (Ψ_L) and leaf water potential measured predawn (Ψ_{PD}), leaf water content and leaf isotopic composition (δ^{13}C). Overall statistical results are shown in Table S4 available as Supplementary Data at *Tree Physiology* online. Averages topped with different letters are significantly different at $P < 0.05$.

in mining damage ($F_{1,62} = 1.27$, $P = 0.26$). Leaf area was not significantly related to RWC ($F_{1,62} = 1.74$, $P = 0.19$). The overall model explained only 26% of the variability in RWC, indicating that additional, unexplained sources of variability contributed heavily to leaf water loss.

Discussion

The epidermal leaf miner *P. populiella* has complicated effects on aspen physiology due to the insect's habit of feeding on both stomatous and astomatous leaf surfaces, leading to different and sometimes opposing effects on leaf function. Both adaxial and abaxial mining damage decreased A, although the effect of abaxial mining was the stronger of the two (Figure 6a), but effects of adaxial and abaxial mining on several leaf variables related to water use differed markedly. Mining of the abaxial leaf surface reduced g_s, Tr and Ψ_L and increased leaf moisture content, most likely due to the impaired function of stomates in mined portions of the leaf. In contrast, we found no evidence that mining damage to the adaxial epidermis affected average g_s, Tr or δ^{13}C (Figure 6), and therefore no evidence of indirect effects by adaxially feeding *P. populiella* on the function of stomata. Rather, adaxial mining caused a reduction in Ψ_L and an overall decrease in leaf water content, suggesting it increases the permeability of the epidermis and cuticle to water vapor. When stomatous surfaces were coated in wax to prevent water loss through stomates, we detected a negative relationship between adaxial mining damage and final RWC of leaves, providing more

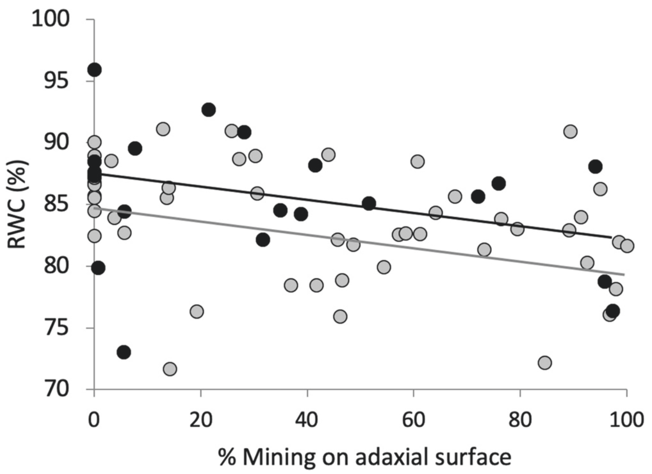

Figure 7. The relationship between mining damage to the adaxial surface and the RWC of detached aspen leaves subjected to a short period at low humidity. Symbol and line colors denote the leaf's site of origin (black, WR; gray, RP).

direct evidence for an increase in cuticular permeability due to epidermal mining.

Folivory by insects generally decreases photosynthesis (Zhou et al. 2015), but effects on water relations can differ widely among functional groups and taxa. Damage by ectophagous insects can greatly increase the rate of water loss from chewed surfaces for days following the damage (Aldea et al. 2005). In contrast, several leaf miners, including taxa that feed on mesophyll and therefore do not directly damage epidermal tissues, reduce g_s and *Tr* (Whittaker 1994, Haile and Higley 2003, Raimondo et al. 2003) and may even increase WUE (Pincebourde et al. 2006). In contrast, epidermal mining by *P. populiella* reduced g_s and *Tr* only when it occurred on the abaxial surface of aspen leaves, and despite this, natural levels of feeding damage, which occur on both leaf surfaces, resulted in lower leaf moisture overall. Similarly, epidermal leaf mining by *P. citrella* on *Avicenna marina* leaves also increases the rate of leaf water loss (Chen et al. 2017).

Although leaf mining by *P. populiella* increased the permeability of the epidermis to water vapor, there was no evidence it increased permeability to CO_2. Specifically, we did not detect a depletion of $\delta^{13}C$ in adaxially mined leaves relative to undamaged leaves, as might be expected if, like H_2O, CO_2 diffused more effectively across the mined than the unmined adaxial cuticle, providing a greater opportunity for discrimination against ^{13}C (O'Leary 1993). One explanation for the observed result is that plant cuticles are relatively impermeable to CO_2. Studies on several plant species indicate that water vapor diffuses 20- to 40-fold faster than CO_2 through the epidermis and cuticle (Boyer 2015a, Tominaga et al. 2018). As a result, when gas transport is dominated by movement through the cuticle, such as across an astomatous surface or when stomata are partially closed, transport of water vapor is affected to a greater degree than CO_2 (Boyer 2015*b*). This difference in the diffusivity of H_2O and CO_2 through the cuticle complicates our ability to use data on intercellular CO_2 concentration (C_i) to understand the mechanism for reduced photosynthesis by mined leaves. As suggested by Wagner et al. (2008), a likely mechanism for reduced photosynthesis in abaxially mined leaves is intercellular CO_2 limitation resulting from mining-related closure of stomata. However, the calculation of C_i is based on the movement of water vapor and assumes that both gases move through the stomata rather than the cuticle, with water moving only 1.6 times faster than CO_2 (Boyer 2015*b*). Calculated values can therefore overestimate C_i when gas exchange occurs across the cuticle, such as when the stomata are partially closed (Boyer 2015*a*). This implies measurements of C_i for mined leaves made using standard methods such as we employ in this study are unreliable. An additional mechanism by which mining might reduce photosynthesis is the degradation of chloroplasts within the mesophyll, such as was observed in leaves damaged by the epidermal miner *Phyllocnistis citrella* (Chen et al. 2017). This mechanism is also consistent with our observation that mining restricted to the adaxial surface reduced *A* without altering stomatal function. Further work will be necessary to detail the mechanisms by which *P. populiella* reduces photosynthesis in aspen leaves.

We suspect that the mining-related increase in cuticular permeability we detected by measuring water loss across variably mined adaxial surfaces applies to abaxial surfaces as well; in fact, the rate of water loss across mined regions of the abaxial surface may exceed that of the adaxial surface for several reasons. Imperfectly closed stomatal pores within the epidermal tissue of mined leaves could provide a path for water vapor loss (Kerstiens 1996). In addition, cuticular membranes of guard cells may have lower resistance to the diffusion of water than epidermal cells (Šantrůček et al. 2004). However, effects of leaf mining damage to the abaxial surface were clearly dominated by the effects of the closed and unresponsive stomata during periods conducive to photosynthesis, resulting in reduced g_s and *Tr* relative to unmined or adaxial-mined leaves, as well as higher leaf water retention and a greater propensity to fix ^{13}C.

Under natural conditions, aspen leaves typically sustain mining damage by *P. populiella* to both surfaces, and we found the net effect of leaf mining on water relations to be negative. Relative to insecticide-treated ramets, naturally mined trees had lower WUE, Ψ_{PD} and leaf water content. This suggests that the effect of epidermal mining on aspen water relations is dominated by water loss from the adaxial surface when conditions are permissive to photosynthesis, and perhaps from both surfaces during times not conducive to photosynthesis. Two factors likely contribute to this pattern. First, relative to the abaxial leaf surface, the adaxial surface typically receives more direct solar radiation. Second, average leaf mining damage by *P. populiella* to the adaxial leaf surface generally exceeds damage to the abaxial surface in juvenile trees such as those used in this study (e.g., Figure 3; Doak et al. 2007, Wagner et al. 2008).

We hypothesized that negative effects on leaf physiology would increase throughout the growing season, due to the accumulation of incidental damage to mined epidermal tissue. We found no change in the effect of insecticide treatment on foliar $\delta^{13}C$ over the season, which might be explained by low carbon turnover in leaf tissue following leaf expansion, regardless of accumulated damage. However, we also found no change over time in the magnitude of the treatment effect on variables that would be expected to change in response to accumulated perforation of the mined epidermis, such as *Tr*, Ψ or leaf water content, and therefore no evidence that the negative impact of leaf mining damage by *P. populiella* intensified across the growing season. Rather, results suggest that mining damage to the epidermis changes its properties with respect to permeability to water vapor shortly after the damage occurs, leading to compromised function throughout the remaining lifespan of the leaf, and that accumulated damage to the mined epidermis, if it occurs, does not substantially change the overall effect of leaf mining over time.

Conclusions

The influence of epidermal mining damage by *P. populiella* on the water relations of aspen leaves reflects a balance between two mechanisms with opposing effects: damage to the abaxial leaf surface reduces water loss via stomatal transpiration, while damage to the adaxial surface increases water loss via cuticular transpiration. The net effect of natural levels of leaf damage by *P. populiella*, which can occur on either side but is more prevalent on the adaxial surface, is to increase water loss while decreasing carbon assimilation. The negative impact of *P. populiella* on the water relations of aspen leaves is significant in the context of climate change. Throughout the western portion of its range, large-scale declines in aspen productivity have been linked to drought (Hogg et al. 2008, Michaelian et al. 2011, Chen et al. 2018) and attributed chiefly to hydraulic failure (Anderegg et al. 2012, 2013, Adams et al. 2017). By compromising the water-retaining properties of the leaf, infestations by *P. populiella* within the boreal forest may intensify the negative effects of high vapor pressure deficit at the level of the leaf (Hogg et al. 2000, Trugman et al. 2018), exacerbating the stresses of a rapidly warming environment on aspen productivity and performance.

Supplementary Data

Supplementary Data for this article are available at *Tree Physiology* Online.

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Authors' contributions

S.J.B. and D.W. conceived the experimental design and approach to data collection, with contributions from J.M.W. All co-authors participated in the implementation of experiments and collection of data. D.W. took the lead on data analysis and interpretation. D.W. and J.M.W. wrote the initial manuscript, which all co-authors edited and revised.

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