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CO_2-mediated changes in aspen chemistry: effects on gypsy moth performance and susceptibility to virus

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Abstract

We investigated the effects of long-term CO_2 enrichment on foliar chemistry of quaking aspen (*Populus tremuloides*) and the consequences of chemical changes for performance of the gypsy moth (*Lymantria dispar*) and susceptibility of the gypsy moth to a nucleopolyhedrosis virus (NPV). Foliage was collected from outdoor open-top chambers and fed to insects in a quarantine rearing facility. Under enriched CO_2, levels of leaf nitrogen declined marginally, levels of starch and phenolic glycosides did not change, and levels of condensed tannins increased. Long-term bioassays revealed reduced growth (especially females), prolonged development and increased consumption in larvae fed high-CO_2 foliage but no significant differences in final pupal weights or female fecundity. Short-term bioassays showed weaker, and sex-specific, effects of CO_2 treatment on larval performance. Correlation analyses revealed strong, negative associations between insect performance and phenolic glycoside concentrations, independent of CO_2 treatment. Larval susceptibility to NPV did not differ between CO_2 treatments, suggesting that effects of this natural enemy on gypsy moths are buffered from CO_2-induced changes in foliar chemistry. Our results emphasize that the impact of enriched CO_2 on plant–insect interactions will be determined not only by how concentrations of plant compounds are altered, but also by the relevance of particular compounds for insect fitness. This work also underscores the need for studies of genetic variation in plant responses to enriched CO_2 and long-term population-level responses of insects to CO_2-induced changes in host quality.

Keywords: elevated CO_2, herbivory, *Lymantria dispar*, *Populus tremuloides*, secondary compounds, tritrophic interactions

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Introduction

Trophic interactions and cascades are increasingly recognized as playing pivotal roles in ecosystem dynamics. Of these interactions, herbivory is of singular importance, as it controls the flow of energy and nutrients from plants to higher trophic levels. The process of herbivory is itself strongly affected by the chemical composition of plants, which in turn is influenced by the availability of critical resources (Bryant *et al*. 1983; Coley *et al*. 1985). One such resource is CO_2, levels of which are increasing at a rate of 0.5% per year (Watson *et al*. 1990).

Enriched atmospheric CO_2 may thus alter carbon cycling and ecosystem structure and function not only directly, by affecting plant productivity, but also indirectly, by altering plant chemistry and trophic interactions. Over the last decade, evidence has accumulated that growth under high CO_2 concentrations increases the C:N balance of plant tissues. Levels of foliar starch and nitrogen typically increase and decrease, respectively (Lincoln *et al*. 1993; Watt *et al*. 1995; Lindroth 1996). More recent research with woody plants suggests that levels of phenolics, especially tannins, may also increase (Roth & Lindroth 1995; Lindroth 1996).

Given that variation in concentrations of foliar constituents can influence herbivore performance, CO_2-mediated shifts in plant–insect interactions are likely to occur. A growing number of studies shows this to be the case. CO_2-

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induced shifts in foliar chemistry elicit changes in insect survival, feeding and growth rates, and food utilization efficiencies (Lincoln *et al*. 1993; Roth & Lindroth 1995; Watt *et al*. 1995; Lindroth 1996). The magnitudes of effects on both plants and insects vary, however, in relation to the availability of other required resources (soil nutrients (Kinney *et al*. 1997), water (Roth *et al*. 1997)), and differ among plant and insect species (Watt *et al*. 1995; Lindroth 1996).

To date, nearly all studies on the effects of enriched CO_2 on plant chemistry and insect performance have relied on short-term studies using potted plants (typically grown in environmental chambers) and short-duration bioassays. Such research has been invaluable in providing insight into various patterns of responses. The need is increasing, however, for studies conducted with plants grown under more natural conditions and utilizing bioassays of longer duration. Moreover, exceedingly few studies have considered how CO_2-mediated changes in plant chemistry may alter the dynamics between insects and their natural enemies. Research addressing these considerations is needed to further improve our understanding of the effects of global environmental change on ecological interactions.

The purpose of this research was to investigate the effects of long-term (>1 year) CO_2 enrichment on phytochemistry of quaking aspen (*Populus tremuloides*) and consequences for performance of the gypsy moth (*Lymantria dispar*) and susceptibility of the gypsy moth to nucleopolyhedrosis virus (NPV). Quaking aspen is a fast-growing, early successional species comprising a major forest type in the Great Lakes region of North America. The gypsy moth is an outbreak species, has recently become established in the Lakes region, and utilizes aspen as a preferred host species. NPV is an important natural enemy of gypsy moths and can cause extensive mortality during insect population outbreaks.

Earlier research with this experimental system showed that aspen foliar chemistry, especially concentrations of phenolic glycosides, strongly influences gypsy moth performance (Hemming & Lindroth 1995; Lindroth & Hwang 1996a; Hwang & Lindroth 1997). Aspen tannins have little direct effect on gypsy moths (Hemming & Lindroth 1995) but high concentrations appear to reduce gypsy moth susceptibility to NPV (Lindroth & Hwang 1996a). Aspen chemistry can vary markedly among trees, in response to both environmental and genetic factors (Lindroth & Hwang 1996a; Hwang & Lindroth 1997). Finally, short-term (2 months) CO_2 enrichment studies revealed that aspen phytochemistry responds to CO_2 levels, and, depending on the response of phenolic glycosides, may alter performance of gypsy moths (Lindroth *et al*. 1993; Roth & Lindroth 1995; Kinney *et al*. 1997). Based on this information, we developed the following hypotheses:

H1 Enriched atmospheric CO_2 will decrease levels of nitrogen but increase levels of starch, phenolic glycosides and/or tannins, in aspen leaves.

H2 Performance of larvae reared on high CO_2 foliage will decline, as indicated by prolonged development and decreased growth, fecundity and food utilization efficiencies. Food consumption and nitrogen utilization efficiencies will increase.

H3 Susceptibility to virus in larvae reared on high CO_2 foliage will decrease.

Materials and methods

Plant materials and treatments

Quaking aspen were propagated from a half-sib seedlot acquired from the NorthCentral Forest Experiment Station, Grand Rapids, MN, USA. Seeds were germinated in a glasshouse in April 1994, and in early May germinants were transplanted to eight large (4.7 m diameter, 3.6 m height) open-top chambers located at the West-Madison Experimental Farm (University of Wisconsin, Madison, WI, USA.) The chambers were situated on a former alfalfa field; the soil at this site is a relatively fertile, typic arguidoll (Hole 1976). Trees were watered daily throughout the study.

Chambers were covered with clear, 0.02 cm polyvinyl chloride film containing UV inhibitors (Livingston Coating Co., Charlotte, NC, USA), and no raincap was installed. On average, with respect to ambient conditions, photosynthetic photon flux density (PPFD) was reduced by 12% inside the chambers, whereas air temperature was increased by 2.1 °C. Ambient air was pumped through the chambers at a rate of 3 $m^3\ s^{-1}$, providing about three complete air exchanges per minute. In four of the chambers, atmospheric CO_2 concentration was continuously elevated by an average of 283 μmol mol^{-1} (s.e. = 12 μmol mol^{-1}, based on $n = 336$ instantaneous samples) throughout the 1994 and 1995 growing seasons (May through September). Daytime CO_2 concentrations in the ambient and elevated CO_2 chambers averaged 357 and 640 μmol mol^{-1}, respectively, during the study. CO_2 was supplied from a pressurized 12-ton receiver (Liquid Carbonic CO_2 Co., Woodridge, IL, USA).

Trees were grown under the experimental conditions for the 1994–95 field seasons. Experiments reported here commenced in May 1995, following leaf flush but prior to full leaf maturation. At that time, aspen trees were 2–3 m in height.

Foliar chemistry

Leaf samples for chemical analyses were collected midway through the fourth instar feeding bioassay and at the start of the NPV bioassay (see below). For each sample, ≈ 2 g

of leaves were collected from throughout the canopy of a tree. Samples were stored on ice and returned to the lab, where they were frozen in liquid nitrogen, freeze-dried, ground and stored (–20 °C). We analysed the samples for constituents likely to influence insect feeding (nitrogen, starch, phenolic glycosides) and gypsy moth susceptibility to NPV (phenolic glycosides, condensed tannins).

Foliar nitrogen was measured by Kjeldahl analysis. Digestions were conducted according to the method of Parkinson & Allen (1975), and nitrogen concentrations were quantified by the micro-Nesslerization technique of Lang (1958). Glycine *p*-toluene-sulphonic acid (5.665% N) served as a nitrogen standard. For starch analyses, we used the enzymatic method of Schoeneberger *et al*. (unpublished method), as described by Lindroth *et al*. (1993). The major secondary compounds in aspen are phenolics, including phenolic glycosides and condensed tannins (Palo 1984; Lindroth *et al*. 1987; Lindroth & Hwang 1996a). The dominant phenolic glycosides are salicortin and tremulacin; these were quantified via high performance thin layer chromatography (Lindroth *et al*. 1993). For condensed tannin analyses, leaf tissue was first extracted in 70% acetone (with 10 mM ascorbic acid as antioxidant) at 4 °C. Extracts were then subjected to the butanol-HCl procedure of Porter *et al*. (1986) for quantification of condensed tannins. Condensed tannins purified from quaking aspen (technique of Hagerman & Butler 1980) served as the reference standard.

Insect bioassays

We obtained first instar gypsy moths (New Jersey standard strain) from the Otis Methods Development Centre (Otis Air National Guard Base, Massachusetts, USA). We reared larvae on artificial diet (ODell *et al*. 1985) for stadia 1–2, before initiation of bioassays. Because Wisconsin quarantine restrictions preclude use of gypsy moth in field studies, our bioassays were performed with foliage clipped from trees in the open-top chambers and fed to gypsy moths in a quarantine insect rearing facility. Except as noted below, all rearing was done in a Percival environmental chamber set at 25:21 °C on a 15:9 light–dark cycle.

We conducted two types of bioassays of aspen foliage. Our long-term growth assay monitored larval growth and development (third through fifth stadia), final pupal weights and adult fecundity. Our short-term performance assay (fourth instars) measured detailed, individual consumption and growth rates, and food utilization efficiencies. For both bioassays, the overall experimental design included four chambers at each of two CO_2 levels, two trees (subsamples) within each chamber, and multiple insects (sub-subsamples) per tree.

For the long-term growth assays, 10 newly molted third instars were placed into a plastic rearing container and fed a cluster of aspen leaves clipped from an experimental tree. Leaf petioles were inserted into water piks, and leaves were replaced at 1–2 day intervals. We assayed one set of 10 larvae for each of two trees per open-top chamber. Larvae were maintained under the environmental conditions described previously, except for a 10-day period late in the study when the temperature regime was accidentally reset to a constant 21 °C. Larval weights were recorded at 4–5 day intervals, and pupal sex, weights and development times were recorded 2–3 days following pupation. Total frass production was measured as an index of leaf consumption. Each set of pupae was then placed into a plastic 'shoebox' container with brown paper covering the sides. Upon eclosion, adults mated and females deposited egg masses onto the paper. Egg masses were then dehaired, and eggs counted and weighed.

Prior to initiation of the short-term performance assays, newly molted third instars were reared in groups of 25 on ambient or elevated CO_2 foliage. Upon moulting into the fourth stadium, larvae were weighed and placed individually into petri dishes containing excised, weighed aspen leaves. Leaves were hydrated and replaced as described previously. We assayed three larvae for each of two trees per open-top chamber. Upon moulting into the fifth stadium, larvae were frozen, then larvae, frass and uneaten leaves were freeze-dried and weighed. We calculated nutritional indices based on standard formulas (Waldbauer 1968; Table 1), except that initial rather than average larval weights were used to calculate relative growth and consumption rates (Farrar *et al*. 1989). Initial dry weights of larvae were determined using wet:dry weight conversions derived from a sample of 20 newly molted fourth instars. Dry weight conversions for leaves were based on wet:dry weight ratios of leaf samples taken for chemical analyses.

Table 1 Nutritional indices for fourth instar performance trials*

Dur	Duration of stadium
RGR	Relative growth rate = biomass gained/[(initial larval biomass)(day)]
RCR	Relative consumption rate = food ingested/[(initial larval biomass)(day)]
TC	Total consumption = food ingested
AD	Approximate digestibility = [(food ingested – frass)/food ingested] × 100
ECD	Efficiency of conversion of digested food = [biomass gained/(food ingested-frass)] × 100
ECI	Efficiency of conversion of ingested food = (biomass gained/food ingested) × 100
RNAR	Relative nitrogen accumulation rate = biomass nitrogen gained/[(initial larval biomass)(day)]
RNCR	Relative nitrogen consumption rate = biomass nitrogen ingested/[(initial larval biomass)(day)]
NUE	Nitrogen utilization efficiency = (biomass nitrogen gained/nitrogen ingested) × 100

*Weights expressed as mg dry weight.

Nitrogen utilization parameters were calculated following Kjeldahl nitrogen analyses (see *Foliar chemistry*) of leaves and caterpillars.

NPV pathogenicity assays

Clean NPV solution (4.98×10^8 polyhedral inclusion bodies/mL in phosphate buffer) was obtained from the U.S. Forest Service, Northeastern Forest Experiment Station (Hamden Connecticut, USA). This stock solution was diluted with 1% aqueous Plyac® (a spreader/sticker formulation) to produce concentrations that would deliver 15,000, 30,000 and 60,000 PIB/μl; 1% aqueous Plyac® served as a control.

We conducted bioassays for each of the four concentrations (control + 3 NPV) using foliage from each of two aspen trees, in each of four chambers per CO_2 treatment. (Trees used for this study were different from those used for the insect bioassays previously described.) For each tree assayed, we punched ≈ 100 leaf disks (6.5 mm diameter) from a cluster of leaves. From these, we placed a single leaf disk into each well of three 24-well tissue culture plates, and into 12 wells of a fourth plate. Leaf disks in plates 1–3 were then inoculated with 15,000, 30,000 or 60,000 PIB NPV in a 1 μL droplet; disks in the fourth plate received the control solution. Single newly molted third instars were added to each well. Plates were covered with a damp paper towel (to keep leaf disks hydrated) and sealed.

Larvae were allowed to feed for 24 h, by which time > 90% of the leaf disks were consumed. Larvae that had consumed entire disks were then transferred individually to 30 mL plastic cups containing artificial diet, and maintained at room temperature for 14 days. At that time we recorded percentage mortality for each set of ≈ 12 (control) or ≈ 24 (NPV treatments) larvae. All dead larvae exhibited standard symptoms of NPV pathogenicity (darkened, moist, disintegrating cuticles).

A portion of each leaf sample collected for NPV assays was designated for chemical analysis. We measured levels of phenolic glycosides and condensed tannins as described previously (*Foliar chemistry*). (These leaves were collected approximately 10 days earlier than those collected for the gypsy moth feeding bioassays.)

Statistics

The SAS statistical package was used for analysing data (SAS Institute 1989). Due to the low level of replication of the CO_2 treatment, the probability of Type II statistical errors was high. Thus, in this paper we relax the range of *P*-values accepted as significant, referring to values in the range 0.050–0.100 as 'marginally significant.'

Phytochemical data were analysed by *t*-tests (α = 0.05). Data from each tree within a chamber were treated as subsamples, whereas chambers were treated as true statistical replicates.

For the long-term insect growth trials, results for development time, pupal weight and female fecundity were analysed by *t*-tests (sexes separate). Data for individual larvae (per tree) were treated as subsubsamples, for individual trees (per chamber) as subsamples, and for individual chambers (per CO_2 treatment) as true replicates. Correlation analyses were conducted to identify relationships between insect performance parameters and foliar chemical characteristics.

We used analysis of variance (split-plot model) for statistical analysis of results from the fourth instar performance assay. When overall larval growth is good, as was the case in this study, sex-related differences in performance may occur in fourth instars. The split-plot model was:

$$Y_{ijk} = \mu + C_i + Ch_j(C_i) + S_k + (CS)_{ik} + \varepsilon_{ijk},$$

where Y_{ijk} represents the average insect response over CO_2 level i, chamber j, and sex k.

Fixed effects consisted of CO_2 level (C_i), sex (S_k), and the $CO_2 \times$ sex interaction [$(CS)_{ik}$]. Whole plot and subplot errors were represented by chambers nested within CO_2 [$Ch_j(C_i)$] and ε_{ijk}, respectively. *F*-tests were conducted for the CO_2 effect with $Ch_j(C_i)$ as the error term ($F_{1,6}$), whereas *F*-tests for sex and $CO_2 \times$ sex were conducted with ε_{ijk} as the error term ($F_{1,6}$). Analysis of covariance has been suggested to be more appropriate than use of ratio variables for analysis of nutritional indices (Raubenheimer & Simpson 1992). We present our results in the traditional (ratio) form because SAS does not accomodate straightforward covariate analysis within a split-plot model, and because the regression approach we used previously (Roth & Lindroth 1994) was inappropriate for this study due to the low amount of replication within a subplot (sex within chamber). Moreover, related research (Hwang & Lindroth 1997) has shown that the ratio- and nonratio-based forms of analysis give essentially identical results when a narrow range of initial insect sizes is used, and use of the traditional form afforded direct comparison of results from this study with those of earlier studies. Correlation analyses of insect performance and foliar chemistry were conducted as described previously.

Finally, results from the NPV pathogenicity assays were analysed with *t*-tests (α = 0.05). For each NPV dose, percentage mortality data for each tree (within a chamber) were treated as subsamples; average responses per chamber were treated as true statistical replicates. Correlation analyses were conducted to identify relationships between larval mortality from NPV and foliar chemistry.

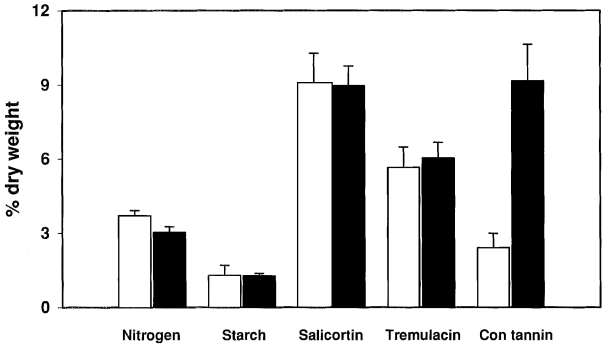

Fig. 1 Chemical composition of foliage used for insect performance trials; trees grown under ambient (light bars) or elevated (dark bars) CO_2 conditions. Vertical lines indicate 1 SE. Con tannin, condensed tannin. Results of statistical analyses are presented in Table 2.

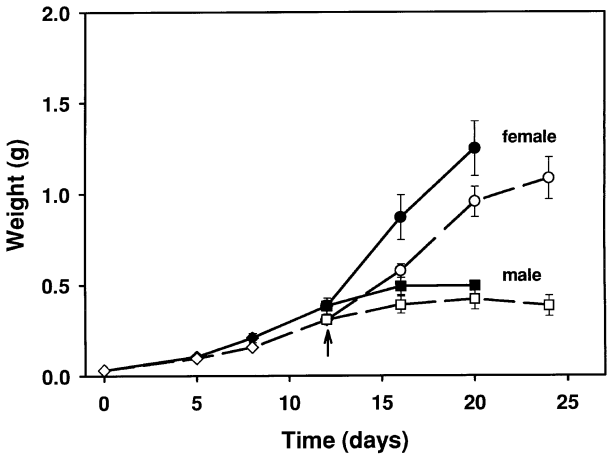

Fig. 2 Growth of gypsy moths reared on leaves from aspen trees grown under ambient or elevated CO_2 conditions. Data recorded from molt into third stadium to onset of pupation; note that insects fed elevated CO_2 leaves required more time to begin pupating. Filled symbols indicate ambient CO_2 foliage; open symbols indicate elevated CO_2 foliage. Arrow shows point at which larval sexes could be determined. Vertical lines indicate ± 1 SE.

Results

Foliar chemistry

CO_2 treatment altered concentrations of several foliar constituents (Fig. 1, Table 2). The 18% decline in nitrogen concentrations under enriched CO_2 was only marginally significant. Levels of starch and of the phenolic glycosides salicortin and tremulacin were unaffected by CO_2. Condensed tannin concentrations, however, increased 3.8-fold under high CO_2.

Insect bioassays

Long-term growth assay. For the first half of the long-term feeding bioassay, larval weights varied little between insects fed ambient- or enriched-CO_2 foliage (Fig. 2). After 12 days, however, weights diverged between the two treatments, with insects growing slower on high-CO_2 foliage. Growth suppression due to CO_2 treatment was greater for females than for males. Development times for females and males reared on high-CO_2 foliage were extended by 9 and 12%, respectively, with the result that final pupal weights did not differ significantly between insects in the two CO_2 treatments (Table 3). Frass accumulation indicated that consumption by insects fed high-CO_2 foliage was substantially higher than for insects fed ambient foliage (Table 3).

Female fecundity was not affected by CO_2 treatment (Table 3). Although both the average number of eggs per egg mass and average egg weight tended to decline for insects reared on enriched-CO_2 leaves, substantial within-treatment variation obscured possible treatment differences.

Correlation analyses reveal potential relationships between insect performance and phytochemistry, independent of CO_2 treatment (Table 4). Performance parameters were consistently and strongly associated with foliar concentrations of phenolic glycosides, but not with levels of other aspen constituents. Reduced insect performance was associated with high levels of phenolic glycosides, as revealed by positive correlations for development time and negative correlations for pupal weight and fecundity.

Short-term performance assay. Aspen CO_2 treatment had relatively few effects on performance of fourth instars, and these effects generally differed between males and females (Table 5). Duration of the fourth stadium did not differ between CO_2 treatments or sexes. Enriched CO_2 led to an 18% increase in relative growth rates (RGRs) of females but to a 13% decline in RGRs of males, a marginally significant $CO_2 \times$ sex interaction. Relative consumption rates (RCRs) showed a small (14%) and marginally significant increase under high CO_2, whereas total consumption during the stadium did not differ among insects in the two CO_2 treatments. Approximate digestibility (ADs) of leaf tissue declined by 23% in females fed high-CO_2 foliage but by only 7% in males fed such foliage, a marginally significant interaction effect. The efficiencies with which aspen tissue was converted into insect biomass (ECDs, ECIs) were not affected by CO_2 treatment. Insect nitrogen budgets, however, were influenced by CO_2 treatment and sex. Relative nitrogen accumulation rates (RNARs) increased 15% in females reared on high-CO_2 foliage, but decreased 10% in males reared similarly (a marginally significant $CO_2 \times$ sex interaction). RNARs are the product of relative nitrogen consumption rates (RNCRs) and nitrogen utilization effi-

Table 2 Summary of statistical analyses (t-tests): effects of enriched CO_2 on aspen chemistry

Bioassay/statistic	Nitrogen	Starch	Salicortin	Tremulacin	Condensed tannins
Feeding bioassays:					
t (6 d.f.)	2.15	0.06	0.09	0.36	4.27
P	0.076	0.956	0.930	0.734	0.005
NPV bioassays:					
t (6 d.f.)	0.35	1.56	1.80		
P	–	–	0.736	0.170	0.122

Table 3 Long-term gypsy moth performance on ambient- and elevated-CO_2 aspen foliage ($\chi = \pm 1$ SE).

	Development time (d)[a]		Pupal weight (g)				
Treatment	Female	Male	Female	Male	Frass production (mg)	Fecundity Eggs/female	Egg weight (mg)
Ambient CO_2	27.8 ± 0.4	23.5 ± 0.6	1.019 ± 0.076	0.409 ± 0.019	619 ± 33	366 ± 54	0.64 ± 0.04
Elevated CO_2	30.4 ± 0.4	26.2 ± 0.5	0.866 ± 0.057	0.344 ± 0.030	854 ± 80	322 ± 28	0.59 ± 0.03
t (6 d.f.)	3.26	2.33	1.11	1.30	2.72	0.663	0.806
P	0.017	0.059	0.316	0.241	0.034	0.532	0.451

[a]Time elapsed from second larval molt (third stadium) to pupation.

Table 4 Summary of correlation analyses: long-term performance parameters vs. total phenolic glycoside concentrations.

	Development time (d)[a]		Pupal weight (g)		Fecundity	
Statistic	Female	Male	Female	Male	Eggs/female	Egg weight
r^a	0.675	0.657	–0.711	–0.435	–0.831	–0.659
P	0.004	0.006	0.002	0.092	< 0.001	0.006

[a]r = correlation coefficient

ciencies (NUEs). Thus, variation in RNARs reflected a sex-related difference in RNCRs (females consumed nitrogen more rapidly than did males) and sex- and CO_2 × sex-related differences in NUEs. Nitrogen use efficiencies increased 22% in females reared on high-CO_2 foliage but were not altered by CO_2 treatment among males.

Correlation analyses showed that fourth instar performance was associated with variation in aspen chemical constituents, independent of CO_2 treatment (data shown only for phenolic glycosides, Table 6). Consumption rates of both sexes and NUEs of females were negatively correlated with leaf nitrogen concentrations. Growth and consumption rates of males tended to be positively associated with leaf starch concentrations. Consumption rates and NUEs tended to be positively correlated, whereas ADs were negatively correlated, with condensed tannin concentrations. Overall, however, the most consistent and striking correlations existed between insect performance parameters and phenolic glycoside concentrations (Table 6). Stadium duration was positively correlated with phenolic glycoside levels, whereas growth rates, consumption rates, feeding efficiencies (ECDs and ECIs), RNARs and NUEs were negatively correlated with phenolic glycoside levels.

NPV pathogenicity assays

Results from our chemical analyses of leaves used in the NPV assays show that CO_2 treatment had no significant effect on levels of foliar phenolic glycosides (salicortin and tremulacin) or condensed tannins (Fig. 3, Table 2). A potential CO_2 effect for condensed tannins (77% increase in high-CO_2 plants) may have been obscured by substantial within-treatment variation and small sample sizes.

Gypsy moth mortality from NPV increased with viral dose but was not altered by aspen CO_2 treatment (Fig. 4). Moreover, correlation analyses (results not shown)

Table 5 Short-term (fourth instar) gypsy moth performance on ambient- and elevated-CO_2 aspen foliage ($\bar{x} \pm 1$ SE)[a].

Treatment	Sex	Dur (d)	RGR (mg/mg/d)	RCR (mg/mg/d)	TC (mg)	AD (%)	ECD (%)	ECI (%)	RNAR (mg/mg/d)	RNCR (mg/mg/d)	NUE (%)
Amb.CO_2	Female	7.21 ± 0.52	0.487 ± 0.046	3.17 ± 0.18	239.7 ± 9.93	41.87 ± 2.26	37.12 ± 4.30	15.11 ± 0.97	0.060 ± 0.005	0.117 ± 0.011	50.86 ± 2.05
	Male	6.46 ± 0.67	0.411 ± 0.057	2.65 ± 0.31	177.9 ± 32.5	40.16 ± 4.02	39.97 ± 7.14	15.30 ± 1.61	0.050 ± 0.006	0.095 ± 0.006	51.48 ± 4.21
Elev.CO_2	Female	6.89 ± 0.12	0.575 ± 0.029	3.86 ± 0.14	273.0 ± 14.1	32.24 ± 2.49	44.65 ± 2.91	15.00 ± 0.71	0.069 ± 0.002	0.114 ± 0.010	62.27 ± 5.90
	Male	6.87 ± 0.68	0.356 ± 0.015	2.82 ± 0.15	171.1 ± 19.5	37.30 ± 3.01	36.70 ± 5.21	12.66 ± 1.31	0.045 ± 0.002	0.088 ± 0.005	50.26 ± 2.68

[a]See Table 1 for description of parameters.

Table 6 Summary of correlation analyses: short-term performance parameters vs. total phenolic glycoside concentrations.

Sex	Statistic	Dur	RGR	RCR	TC	AD	ECD	ECI	RNAR	RNCR	NUE
Female	r	0.749	–0.798	–0.578	–0.448	0.262	–0.611	–0.674	–0.758	–0.336	–0.514
	P	0.002	< 0.001	0.030	0.108	0.388	0.027	0.008	0.002	0.240	0.060
Male	*r*	0.806	–0.792	–0.552	0.220	0.400	–0.716	–0.717	–0.771	–0.431	–0.820
	P	0.002	0.002	0.062	0.492	0.198	0.009	0.009	0.003	0.162	0.001

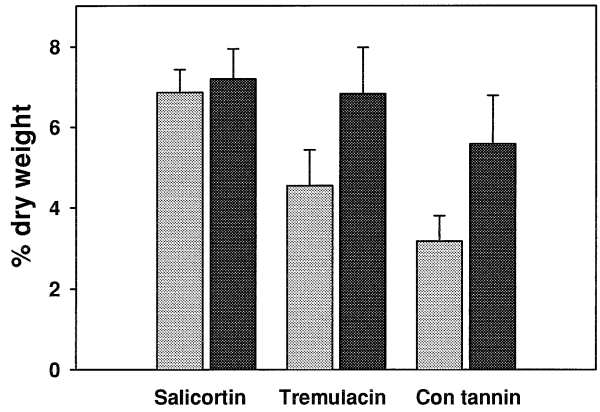

Fig. 3 Concentrations of phenolic glycosides and condensed tannins (con tannin) in foliage used for NPV pathogenicity assays; trees grown under ambient (light bars) or elevated (dark bars) CO_2 conditions. Vertical lines indicate 1 SE. Results of statistical analyses are presented in Table 2.

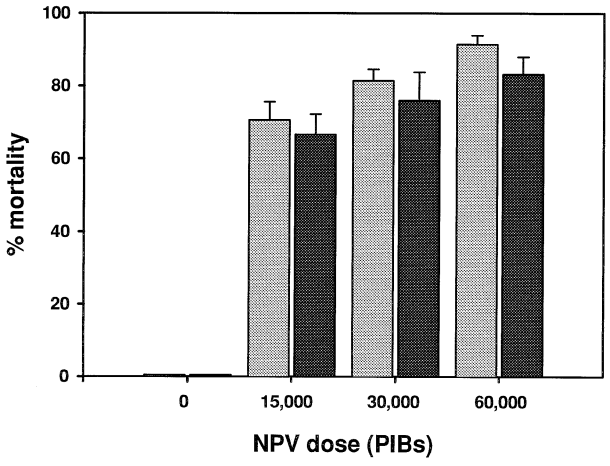

Fig. 4 Gypsy moth mortality due to NPV when fed foliage from aspen grown under ambient (light bars) or elevated (dark bars) CO_2 conditions. Vertical lines indicate 1 SE. *t*-tests revealed no significant CO_2 effects (for the 15,000, 30,000 and 60,000 PIB doses, respectively, $t = 0.55$, $P = 0.603$; $t = 0.65$, $P = 0.542$; $t = 1.52$, $P = 0.180$).

revealed no significant associations between mortality rates and foliar phenolic glycoside or condensed tannin concentrations, independent of CO_2 treatment.

Discussion

Foliar chemistry

According to H1, we anticipated that levels of nitrogen would decrease, while levels of starch, phenolic glycosides and/or condensed tannins would increase, in aspen grown under enriched atmospheric CO_2. Some, but not all, of the predictions were supported by our data. Concentrations of nitrogen declined marginally, whereas those of starch and phenolic glycosides were unaffected and those of tannins nearly quadrupled. Overall levels of nitrogen were high, similar to those of field aspen growing on fertile soils (Lindroth *et al*. 1987; Hemming & Lindroth 1995). The magnitude of decline in nitrogen concentrations was somewhat less than that observed for aspen in our earlier growth chamber studies using nutrient-rich soil media (Lindroth *et al*. 1993; Roth & Lindroth 1995; Kinney *et al*. 1997). The smaller decline may have resulted from lower accumulation of starch, and hence less dilution of foliar nitrogen, in the current study. In our earlier work, starch concentrations ranged from 2 to 18%, with concentrations increasing two- to three-fold under high CO_2. Several explanations exist for the difference in starch accumulation between this and our earlier studies. First, concentrations of foliar starch in previous work with potted aspen may have been artificially high due to root restriction and reduced carbohydrate sink strength (Thomas & Strain 1991; Ceulemans & Mousseau 1994). Secondly, levels of starch in the current study may have been low due to canopy light dynamics. In 1994 (the first year of our 2-year study), aspen seedlings were less than 1 m in height, and foliage contained ≈ 6 and 11% starch in ambient and elevated CO_2 treatments, respectively, (Lindroth, Kruger & Volin, unpublished data). At the time of the current study, however, the canopy had closed, and many of the leaves were shaded. Indeed, foliage collected from a separate portion of our chambers in which aspen were more widely spaced had higher concentrations of starch, which increased significantly with enriched CO_2 (Roth & Lindroth, unpublished data). Additionally, levels of starch in this study were at the low end of the range of values exhibited by unshaded leaves of field aspen (Lindroth & Hwang 1997).

Concentrations of phenolic glycosides (salicortin and tremulacin) were unaffected by CO_2 treatment. Responses

of these compounds to enriched CO_2 have varied in our earlier growth chamber work, with increases, no change and marginal decreases detected in different studies (Lindroth *et al*. 1993; Roth & Lindroth 1995; Kinney *et al*. 1997). The significant increase in condensed tannin levels, however, was consistent with most of our previous research. Reichardt *et al*. (1991) suggested that concentrations of stable end-products of secondary metabolism (tannins) may better reflect variation in resource availability than do concentrations of dynamic (or intermediate) metabolites. Our research and related studies (Kinney *et al*. 1997) indicate that such a pattern holds true for the response of aspen phenolics to high CO_2 concentrations. Finally, overall levels of secondary compounds in our trees were within the range of values found in field aspen, although that range is quite large due to substantial genetic variation in accumulation of phenolics (Lindroth & Hwang 1996b; Hwang & Lindroth 1997).

Insect performance

H2 predicted that for insects fed high-CO_2 foliage, development rates, growth, fecundity and food processing efficiencies would decrease, whereas food consumption and nitrogen use efficiencies would increase. Again, our results supported some, but not all, of the expected trends. Long-term growth rates, especially of females, declined under the high CO_2 treatment. Because these insects prolonged developmental periods by approximately three days, however, final pupal weights approached those of insects fed ambient-CO_2 foliage, and female fecundity was not significantly impaired. Measurement of frass production suggested that over the long-term, consumption rates by larvae on high-CO_2 aspen foliage may increase markedly. Use of this index is complicated by the fact that digestibility of high-CO_2 leaves was lower for larvae (females) on high-CO_2 foliage, and this factor alone accounts for some of the increase in frass production. Nevertheless, given that (i) the magnitude of the difference in frass production between CO_2 treatments was large, (ii) consumption by females exceeded that by males, and (iii) the ratio of females to males was lower in the elevated than in the ambient CO_2 treatment, the difference in food consumption between insects in the two treatments was probably real. This result is consistent with those of numerous other studies that have documented increased feeding by insects on high-CO_2 plants (reviewed by Lincoln *et al*. 1993; Watt *et al*. 1995; Lindroth 1996).

Results from the short-term fourth instar studies showed, overall, weaker insect responses to CO_2 treatment than did the long-term study. Our previous research showed that gypsy moth larvae reared on high-CO_2 aspen typically exhibit prolonged development and increased consumption but reduced growth due to poor food processing efficiencies (Lindroth *et al*. 1993; Roth & Lindroth 1995). Those responses were attributed primarily to phenolic glycoside toxicity; increased concentrations of the compounds in high-CO_2 foliage, and/or accelerated consumption, significantly increased toxic loads on larvae. In this study, however, enriched CO_2 did not alter phenolic glycoside concentrations and consumption rates were only marginally altered, so fourth instar performance was largely unaffected.

Insects may respond to dietary nitrogen limitation not only by altering consumption rates, but by improving nitrogen utilization (Slansky & Feeny 1977; Slansky & Wheeler 1989). Thus H2 also predicted that nitrogen utilization efficiencies would increase for larvae fed nitrogen-dilute, high-CO_2 foliage. Interestingly, we found that NUEs increased only for females on high-CO_2 leaves. This sex-related difference in response may reflect the greater demand for nitrogen (protein) to support more rapid growth and, ultimately, egg production in female larvae. Shifts in NUEs may be expected to be even greater in situations where the CO_2-mediated decline in foliar nitrogen is larger than in this study. In related research, Williams *et al*. (1994) reported an increase in NUEs for pine sawfly reared on high-CO_2 loblolly pine needles, but few other studies have addressed the phenomenon.

Although we found no treatment-associated shifts in phenolic glycoside concentrations, our results illustrate nonetheless the importance of these compounds in interactions between aspen and gypsy moths. Levels of phenolic glycosides varied among individual trees, and this variation was closely matched by variation in insect performance. These results are consistent with correlative and empirical results from several other studies (Hemming & Lindroth 1995; Lindroth & Hwang 1996a; Hwang & Lindroth 1997). This is the first study, however, to suggest that reductions in growth due to phenolic glycosides result in reduced female fecundity. In addition, our results indicate that phenolic glycosides reduce nitrogen utilization efficiencies. The compounds are purported gut toxins (Lindroth & Peterson 1988; Lindroth & Hwang 1996a); reduced NUEs may reflect excessive excretory loss of nitrogen due to degenerative midgut lesions.

Several general conclusions can be drawn from our insect bioassays. First, CO_2-mediated changes in plant chemical composition are likely to have weak effects on insects unless the compounds affected are the very ones primarily responsible for overall insect performance on that plant (protein, toxins). Secondly, changes in insect performance are more easily detected in long-term than in short-term bioassays when phytochemical shifts are relatively small. Finally, this study emphasizes again what has been expressed elsewhere (Lawton 1995; Lindroth *et al*. 1995): a need for long-term, population-level assessments of the effects of enriched CO_2 on plant–insect interactions.

Even relatively minor effects of host quality on individual performance parameters such as survival, development, growth and fecundity can, through multiplicative effects, have significant impact on insect populations.

NPV pathogenicity

Pathogenicity of NPV to gypsy moths is influenced by larval diet, with insects feeding on high-tannin foliage being less susceptible than those feeding on low-tannin foliage (Keating & Yendol 1987; Keating *et al*. 1990; Hunter & Schultz 1993; Lindroth & Hwang 1996a). According to H3, we anticipated that CO_2-mediated increases in condensed tannin concentrations in aspen would reduce larval mortality to NPV. Pathogenicity assays, however, showed that larval mortality was not significantly affected by CO_2 treatment. This result could be explained by the fact that condensed tannin concentrations also were not significantly affected by CO_2 treatment in the NPV study. Nevertheless, the trend toward increased tannin concentrations under high CO_2 was not mirrored by reduced NPV pathogenicity in insects fed high-CO_2 foliage. Moreover, contrary to earlier research (Lindroth & Hwang 1996a), no significant correlation existed between larval mortality due to NPV and aspen tannin concentrations. This discrepancy remains unresolved.

Plant species containing hydrolysable tannins in addition to condensed tannins appear to be more inhibitive toward NPV than those containing only condensed tannins (Keating *et al*. 1990). Thus, CO_2-mediated changes in gypsy moth – NPV interactions may be more pronounced for plant species such as oak and maple, in which hydrolysable tannins as well as condensed tannins respond to CO_2 enrichment (Lindroth *et al*. 1993; Roth & Lindroth 1995; Kinney *et al*. 1997).

Little other research has addressed the effects of CO_2-mediated changes in plant chemistry on natural enemies of insects. Roth & Lindroth (1995) reported that the gypsy moth parasitoid, *Cotesia melanoscela*, was at most only weakly affected by changes in the quality of several tree species, including aspen.

Conclusions

Growth under enriched atmospheric CO_2 alters the foliar chemistry of aspen. Patterns of responses are generally consistent between nonpotted trees in open-top chambers (this study) and potted trees in environmental chambers (earlier research). A major exception is starch, levels of which may be particularly responsive to restrictions in root growth (potted plants) or canopy light dynamics (open-top chambers). Concentrations of nitrogen generally decline. Levels of dynamic secondary metabolites (phenolic glycosides) show variable responses, whereas those of stable secondary end-products (condensed tannins) increase. Given that phenolic glycoside production varies among aspen genotypes, and that different genotypes have been used in our studies, the possibility arises that variation in response to CO_2 is genetically based.

The response of herbivores to CO_2-induced phytochemical changes will depend critically upon which compounds are altered, and the relationship of those compounds to herbivore performance. Phenolic glycosides dominate interactions between aspen and gypsy moths. Because levels of those compounds were not significantly altered by CO_2 treatment in this study, insect performance also was not markedly altered. Our results also suggest that determination of simple C:N ratios in plant tissues is likely to tell us little about how trophic interactions will be affected. Similarly, the consequences of enriched CO_2 for tritrophic interactions will be influenced by how specific chemicals influencing the interactions are affected. At this time the data are few but suggest that such effects will be weak to nonexistent.

Finally, our results highlight several avenues for research at the population level. With respect to plants, intraspecific genetic variation in chemical responses to elevated atmospheric CO_2 should be investigated. Concentrations of secondary metabolites in aspen are influenced by both genetic and environmental factors, and most likely the interplay between them (Lindroth & Hwang 1996a). Exceedingly little is known, however, about how CO_2 may differentially affect the chemical composition of diverse genotypes, and the consequences thereof for herbivores. With respect to herbivores, the effects of multiple changes in individual performance, even when minor, need to be determined at the population level. Such work is essential for evaluating the consequences of future atmospheric composition for trophic interactions and related community dynamics.

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