CO_2 and O_3 Effects on Paper Birch (Betulaceae: *Betula papyrifera*) Phytochemistry and Whitemarked Tussock Moth (Lymantriidae: *Orgyia leucostigma*) Performance

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ABSTRACT Elevated atmospheric concentrations of CO_2 and O_3 are known to alter the chemical composition of foliage, which in turn may affect the performance of herbivorous insects. We investigated the independent and interactive effects of CO_2 and O_3 on foliar quality of paper birch (*Betula papyrifera* Marshall) and the consequences of chemical changes for performance of the whitemarked tussock moth *Orgyia leucostigma* (J. E. Smith). The experimental design was a 2 by 2 factorial, with ambient and elevated levels of CO_2 and O_3, respectively. Foliage was analyzed for concentrations of nitrogen, starch, and condensed tannins. CO_2 and O_3 independently and interactively affected nitrogen concentrations, with the elevated $CO_2 + O_3$ treatment reducing nitrogen concentrations more than either treatment alone. Elevated CO_2 and O_3 had no significant effect on starch and tannin concentrations when administered alone but increased starch concentrations by 17% over ambient when administered together. Larvae were reared on experimental trees from egg hatch through pupation to determine treatment effects on development time and pupal mass. Larval performance measures were not statistically different among fumigation treatments, although females tended to have reduced pupal mass under the elevated $CO_2 + O_3$ treatment. These results demonstrate that chemical responses of some plant species to elevated levels of CO_2 (560 μl L^{-1}) and O_3 (1.5 $\times$ ambient) may be of insufficient magnitude to significantly alter standard measures of individual insect performance.

KEY WORDS *Betula papyrifera*, *Orgyia leucostigma* whitemarked tussock moth, CO_2, Aspen-FACE, O_3

PROJECTIONS INDICATE THAT current concentrations of atmospheric CO_2 will likely double within this century (IPCC 1996) and concentrations of tropospheric O_3 may triple within the next 30 to 40 yr (Chameides et al. 1994). Elevated CO_2 and O_3 have been shown to alter the phytochemistry, physiology, growth, and reproduction of plants (Kohut et al. 1987, Reich 1987, Riemer and Whittaker 1989, Saxe et al. 1998, Norby et al. 1999, Pritchard et al. 1999). Because elevated levels of CO_2 or O_3 can directly affect foliar quality, these pollutants may also alter relationships between plants and herbivorous insects (Bezemer and Jones 1998, Coviella and Trumble 1999, Trumble et al. 1987, Jackson et al. 2000). However, research has largely investigated the independent rather than combined effects of CO_2 and O_3 exposure on plant-insect interactions. The paucity of studies investigating these pollutants in combination limits our ability to predict how plant-insect interactions will be affected under future atmospheric conditions.

Trees exposed to elevated CO_2 typically have a higher foliar carbon to nitrogen ratio than do trees grown under ambient conditions (Watt et al. 1995; Lindroth 1996a, 1996b; Norby et al. 1999). Specifically, CO_2 enrichment increases concentrations of starch and shikimic acid derived allelochemicals (such as condensed tannins) and decreases concentrations of nitrogen (Roth and Lindroth 1994, Kinney et al. 1997). These changes in foliar quality typically increase development time while decreasing growth and fecundity in leaf-chewing insects (Lincoln et al. 1993; Watt et al. 1995; Lindroth 1996a, 1996b; Bezemer and Jones 1998; Coviella and Trumble 1999).

Few studies have been conducted on the effects of O_3 on the foliar quality of deciduous trees and subsequent effects on insect performance. O_3 is known to alter foliar concentrations of proteins, carbohydrates, minerals, vitamins, and phenolic compounds (Riemer and Whittaker 1989). With respect to secondary metabolites, enzymes important to their production can be affected in two ways. First, O_3 can increase allelochemical concentrations by inducing phenylalanine ammonium lyase (PAL), a key enzyme in the shikimic acid pathway (Rosemann et al. 1991, Eckey-Kaltenbach et al. 1994, Kangasjärvi et al. 1994). Alternatively, O_3 can decrease secondary metabolite concentrations by inhibiting ribulose bisphosphate carboxylase/oxygenase (Rubisco), thereby slowing photosynthesis

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and reducing carbon assimilation (Dann and Pell 1989). Such changes in plant secondary metabolite production may affect the performance of leaf-chewing insects. Previous studies demonstrated that insects respond by increasing consumption (Chappelka et al. 1988, Coleman and Jones 1988) and survivorship (Trumble et al. 1987, Jackson et al. 2000), while decreasing development time (Trumble et al. 1987) and reproductive success (Coleman and Jones 1988). With respect to the effects of O_3 on tree-feeding Lepidoptera, only gypsy moths have been investigated, with results showing that performance depends largely on the O_3 concentrations used and tree species studied (Jeffords and Endress 1984, Endress et al. 1991, Lindroth et al. 1993). Information is still lacking on the extent to which O_3 influences tree phytochemistry and, in turn, affects important long-term fitness parameters (e.g., development time and fecundity) for herbivorous insects.

Natural ecological systems are subject not to individual atmospheric pollutants, but rather to complex combinations of them. Thus, a growing emphasis of global change research has been evaluation of the interactive effects of pollutants. Ecophysiological studies conducted with deciduous trees have demonstrated that elevated CO_2 has the potential to reduce the deleterious effects of O_3 stress (Kull et al. 1996, Volin and Reich 1996, Volin et al. 1998, Grams et al. 1999, Karnosky et al. 1999). The reduction of such effects has been attributed to lower stomatal conductance under elevated CO_2 (Volin and Reich 1996, Volin et al. 1998). Still, relatively little is known about the interactive effects of CO_2 and O_3 on plant physiology and biochemistry. In particular, effects on phytochemical concentrations, and resultant effects on insect performance, are poorly understood.

The purpose of this study was to evaluate the effects of CO_2 and O_3 (independently and in combination) on the foliar quality of a deciduous tree species and a generalist insect herbivore under field conditions. Specific objectives were (1) to determine the effects of CO_2 and O_3 on the phytochemistry of paper birch (Betulaceae: *Betula papyrifera* Marshall), and (2) to assess the consequences of CO_2- and O_3-mediated changes in phytochemistry for the performance of whitemarked tussock moth, Lymantriidae: *Orgyia leucostigma* (J. E. Smith), larvae. The ability to conduct experiments under natural field conditions was a limitation for most previous CO_2 and O_3 studies. Free-Air CO_2 Enrichment (FACE) technology is now available to conduct open-air experiments (Hendrey 1992, Hendrey and Kimball 1994), allowing for natural atmospheric mixing, light and humidity profiles, and precipitation. In addition, FACE technology allows an increase of scale unparalleled by any other methodology used for controlling pollutant concentrations.

Paper birch and whitemarked tussock moths were selected for use because they are common species in forests of the north-central United States. Paper birch is an early successional tree species found throughout the northern United States and Canada. It serves as a host plant for the whitemarked tussock moth, a generalist herbivore native to the Great Lakes region. Whitemarked tussock moth larvae have been observed feeding on paper birch near the FACE site in northern Wisconsin (B.J.K., unpublished data).

Materials and Methods

Experimental Design and Set-up. This experiment was conducted at the Aspen Free Air CO_2 Enrichment (Aspen FACE) site located near Rhinelander, WI (W 89.7°, N 45.7°). The site was used for agricultural purposes for over 50 yr, and in 1972 was purchased by the U.S. Forest Service for field research. In 1996 it was converted to use as a FACE research site. For a detailed description of the experimental design and operation of the FACE site, consult Dickson et al. (2000). Briefly, the 32 ha site contains 12 FACE rings (30 m diameter) set up as a 2 by 2 factorial design with three rings receiving supplemental CO_2 (560 μl L^{-1}), three rings receiving supplemental O_3, three rings receiving both supplemental CO_2 and O_3, and three rings receiving ambient air (control rings). The site was divided into three blocks, with each block containing one ring of each treatment. Fumigation with CO_2 and O_3 was conducted only during daylight hours of the growing season. Concentrations of CO_2 and O_3 were monitored in all 12 rings. The elevated CO_2 concentrations employed are $\approx$200 μl L^{-1} above ambient and are based on levels predicted for 50–60 yr in the future (IPCC 1996). Due to the photochemical nature of tropospheric O_3 formation, a standard supplemental O_3 concentration was not environmentally realistic. The O_3 concentrations employed were based on weather conditions, and modified to match O_3 levels realized in urban areas in the western Great Lakes region (Pinkerton and Lefohn 1987, Karnosky et al. 1996). On average, target O_3 concentrations were 90–100 nl L^{-1} on sunny days, 50–60 nl L^{-1} on cloudy days, and no O_3 treatment was applied on cool (<15°C) days. Furthermore, O_3 was not administered when leaf surfaces were wetted from fog, dew, or rain events. Ambient air was blown into the rings for the control treatment. Within each ring, three trees were used for both foliar collections and insect bioassays.

Plant and Insect Material. The paper birches, which were 3 yr old at the time of the study, originated from seed collected in Houghton County, MI (Dickson et al. 2000). Seedlings were planted in the FACE rings in summer 1997 and have been exposed to their respective gas treatments since spring 1998. Tussock moth egg masses were obtained from the Forest Pest Management Institute, Canadian Forest Service (Sault St. Marie, Ontario, Canada). Egg masses were surface-sterilized with a solution containing 0.1% sodium hypochlorite and 1% Tween 80, and then placed into growth chambers (Percival, Boone, IA) (16:8 h and 26:18°C light:dark cycle) until hatch.

Phytochemical Analyses. Leaves used for phytochemical analyses were collected on three dates (3 June, 15 June, and 29 June 1999) from the same trees as used for the insect bioassays. To equalize light levels, branches used for foliar collection were bagged

with the same mesh material as used for insect bioassays. Foliage was selected at the same relative position and sun exposure as the foliage used for insect bioassays. Leaves (2–3 g fresh mass) were excised at the petiole and stored under crushed ice (for up to 4 h from first leaf excision) until they could be flash-frozen with liquid nitrogen and freeze-dried in the laboratory. Samples were then ground and stored at −20°C before analysis. Analyses were conducted to determine concentrations of nitrogen, starch, and condensed tannins. Nitrogen concentrations were determined by high-temperature combustion, followed by thermoconductometric detection (LECO FP528 nitrogen analyzer, St. Joseph, MI). Glycine *p*-toluenesulfonate was used as the nitrogen standard (Hach County, Loveland, CO). Starch concentrations were determined by first separating starch from soluble sugars and then enzymatically hydrolyzing starch to glucose using the Prado et al. (1998) method. To quantify glucose concentrations, we used a modification of the dinitrosalicylic acid method (Lindroth et al. 2001a). Condensed tannin concentrations were measured using the butanol-HCl method of Porter et al. (1986), which hydrolytically converts proanthocyanidins to anthocyanidins. Paper birch condensed tannins were purified from leaves of birch trees located near the FACE site by adsorption chromatography (Hagerman and Butler 1980) and used as the reference standard.

Insect Bioassays. Upon hatching (27 May 1999), 60 larvae were randomly assigned to each of three birch trees within each FACE ring. To reduce mortality associated with transfer and establishment, first instars were reared in ventilated 2.5 by 15-cm Petri dishes and fed leaves excised from their assigned tree. Petri dishes were maintained within the FACE ring so larvae were exposed to the corresponding fumigation treatment. Upon molting into the second stadium, the larvae were divided between two bags (30 larvae/bag) on each tree (totaling 60 larvae/tree and 180 larvae/ring). To prevent substantial defoliation of the trees, the number of larvae in each bag was reduced to 10 randomly selected individuals midway through the third stadium (20 larvae/tree). Duration of the larval development period and pupal mass were measured for each larva that successfully pupated (per bag survivorship was ≈70%). Pupal mass was measured 3 d after pupation.

Statistical Analysis. Analysis of variance (ANOVA; PROC MIXED, Littell et al. 1996) was used for statistical analysis. For analysis of phytochemical data we used a 2 × 2 factorial design with repeated measures. The *a priori* statistical model employed was:

$$Y_{ijkl} = \mu + B_i + C_j + O_k + CO_{jk} + e_{ijk} + D_l + CD_{jl} + OD_{kl} + COD_{jkl} + \epsilon_{ijkl}$$

where Y_{ijkl} was the average response of block i, CO_2 level j, O_3 level k, and date l. CO_2 level (C_j), O_3 level (O_k), date (D_l), and their interaction terms [(CO_{jk}), (CD_{jl}), (OD_{kl}), and (COD_{jkl})] represent fixed effects. Random effects include block (B_i), whole plot error (e_{ijk}) and the subplot error (ϵ_{ijkl}). The use of this model for inference requires the assumption that the block and all other treatments are additive (i.e., that the treatment effects are the same for each block). Because we found that this assumption was not met, we describe the procedure by which this was determined and the corresponding changes necessitated for proper analysis. To explore this assumption we considered the previous model, augmented by terms representing the interaction between each fixed effect and block:

$$Y_{ijkl} = \mu + B_i + C_j + O_k + CO_{jk} + [BC_{ij} + BO_{ik} + BCO_{ijk}] + D_l + CD_{jl} + OD_{kl} + COD_{jkl} + [BD_{il} + BCD_{ijl} + BOD_{ikl} + BCOD_{ijkl}]$$

where e_{ijk} was partitioned into block × CO_2 (BC_{ij}), block × O_3 (BO_{ik}), block × CO_2 × O_3 (BCO_{ijk}) and ϵ_{ijkl} was partitioned into block × date (BD_{il}), block × CO_2 × date (BCD_{ijl}), block × O_3 × date (BOD_{ikl}), and block × CO_2 × O_3 × date ($BCOD_{ijkl}$). We determined, by using likelihood methods integral to PROC MIXED, that one or more of these interaction terms was significant for all response variables (Littell et al. 1996). Thus, *F*-tests were conducted for all main effects with degrees of freedom for error assigned using the Satterthwaite approximation (Milliken and Johnson 1984, Littell et al. 1996). Means and standard errors were calculated using the LSMEANS procedure and are reported for each CO_2 × O_3 × date combination.

For analysis of insect performance, we removed date and added sex to the model (tussock moths are sexually dimorphic). *F*-tests were performed on all main effects in the same manner as analyses for the phytochemical data, with degrees of freedom for error assigned using the Satterthwaite approximation (Milliken and Johnson 1984, Littell et al. 1996). Means and standard errors were calculated using the LSMEANS procedure statement and are reported for each CO_2 × O_3 × sex combination.

Because of the low number of replicates ($n = 3$), we report *P* values < 0.10 as "significant," thereby reducing the probability of type II statistical errors. Exact *P* values for all main effects and interactions are provided for those desiring a more stringent ∝ (Tables 1 and 2).

Table 1. Summary of *P*-values for the effects of CO_2, O_3, and date on phytochemistry

Main effects and interactions	Nitrogen	Starch	Condensed tannins
CO_2	0.085 (3.9)	0.398 (4.0)	0.359 (3.0)
O_3	0.074 (2.7)	0.490 (9.2)	0.344 (2.2)
date	0.006 (10.8)	<0.001 (6.1)	<0.001 (8.0)
CO_2 × O_3	0.093 (5.9)	0.035 (3.8)	0.155 (10.0)
CO_2 × date	0.142 (5.9)	0.650 (7.0)	0.516 (8.0)
O_3 × date	0.623 (6.7)	0.857 (9.2)	0.829 (10.0)
CO_2 × O_3 × date	0.021 (12.8)	0.166 (3.8)	0.267 (10.0)

Satterthwaite degrees of freedom for error are given in parentheses.

Table 2. Summary of *P*-values for the effects of CO_2, O_3, and sex on insect performance

Main effects and interactions	Development time	Pupal mass
CO_2	0.284 (4.1)	0.738 (4.8)
O_3	0.858 (3.8)	0.153 (6.8)
sex	0.002 (2.2)	<0.001 (4.8)
$CO_2 \times O_3$	0.917 (3.7)	0.484 (4.8)
$CO_2 \times$ sex	0.974 (2.2)	0.841 (4.0)
$O_3 \times$ sex	0.904 (3.8)	0.292 (4.8)
$CO_2 \times O_3 \times$ sex	0.575 (3.8)	0.162 (4.0)

Satterthwaite degrees of freedom for error are given in parentheses.

Results

Foliar Chemistry. CO_2 and O_3 fumigation altered aspen phytochemistry (Table 1). CO_2 and O_3 independently and interactively affected nitrogen concentrations (Fig. 1), with the elevated $CO_2 + O_3$ treatment reducing nitrogen concentrations more than either the CO_2 or O_3 treatments alone (27%, versus 14% and 7%, respectively). Nitrogen concentrations also decreased across collection dates, and more strongly so under the enriched CO_2 treatment than elevated O_3, elevated $CO_2 + O_3$, and ambient treatments. Starch concentrations were affected by the interaction of CO_2 and O_3 (Fig. 1). Elevated $CO_2 + O_3$ increased starch concentrations 17% over ambient whereas elevated CO_2 and O_3 had no significant effect on starch concentrations when administered alone (Fig. 1). Average starch concentrations decreased 55% from the first to the last foliar collection (Fig. 1). Concentrations of condensed tannins were unaffected by CO_2 and O_3 treatments (Fig. 1). Tannin concentrations tended to be higher under elevated CO_2, but the difference between treatments was not statistically significant even though the elevated $CO_2 + O_3$ treatment had 35% higher tannin levels than did the control treatment. The CO_2 effect may have been obscured by substantial within-treatment variation. On average, condensed tannin concentrations increased 40% from the first to the last foliar collection.

Insect Performance. Our data reveal that elevated CO_2 and O_3 had little to no effect on tussock moth performance (Table 2; Fig. 2). Duration of the larval development period was uniform across all treatments. Females required ≈8 d more than did males to complete their development. Although not quite statistically significant, pupal mass of insects tended to be smaller for those reared under elevated O_3, especially so for females reared under high O_3 and CO_2 (21% less than for females in ambient conditions). Reflecting the sexual dimorphism of the species, female pupal mass was more than twice that of the males.

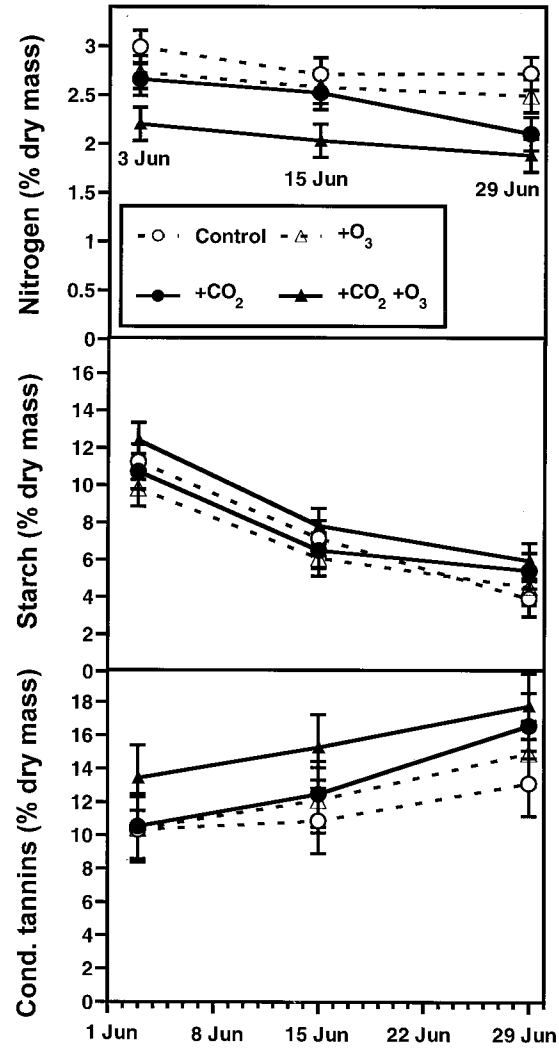

Fig. 1. Concentrations of nitrogen, starch, and condensed tannins under control, elevated CO_2, elevated O_3, and elevated $CO_2 + O_3$ fumigation treatments. Error bars indicate ± 1 SE (calculated from the pooled variance). Cond. tannins, condensed tannins.

Discussion

The consequences of elevated CO_2 and O_3 for foliar quality varied among the phytochemicals measured. In response to elevated CO_2, concentrations of nitrogen declined, whereas those of starch depended on O_3 treatment ($CO_2 \times O_3$ interaction) and levels of condensed tannins were unaffected. The declines in nitrogen concentrations were of similar magnitude to those observed in controlled chamber and glasshouse studies investigating the effects of CO_2 enrichment on paper birch (Roth and Lindroth 1994, Lindroth et al. 1995, McDonald et al. 1999). Reduction of foliar nitrogen concentrations observed in this study was also within the range typically exhibited by other tree species exposed to elevated CO_2 (McGuire et al. 1995, Curtis and Wang 1998, Cotrufo et al. 1998, Norby et al. 1999). Elevated CO_2 and O_3 interacted to affect starch concentrations. The increase in starch concentrations under the combined treatment could be due to O_3 limiting growth (i.e., reduced carbon sink) more than photosynthesis, as suggested by the carbon/nutrient

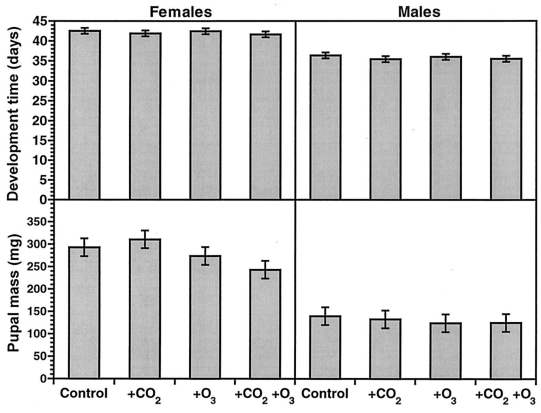

Fig. 2. Tussock moth performance under control, elevated CO_2, elevated O_3, and elevated $CO_2 + O_3$. Error bars indicate ± 1 SE (calculated from the pooled variance).

balance hypothesis (Bryant et al. 1983). In this study, condensed tannin levels did not respond to CO_2 enrichment, a result contrary to previous research that has typically reported CO_2-mediated increases in paper birch tannin levels (Roth and Lindroth 1994, Lindroth et al. 1995, McDonald et al. 1999, Agrell et al. 1999, B.J.K. and R.L.L. unpublished data). Several factors may have contributed to the low magnitude of response of tannin levels in this study. First, the response of tannin levels may have been low due to the CO_2 concentration used in this study ($\approx$560 μl L^{-1}) compared with those used in previous studies (650–700 μl L^{-1}). Second, levels of tannins have been shown to be less responsive to elevated CO_2 under conditions of high nutrient availability, such as exist at the FACE site (Dickson et al. 2001), than under conditions of low nutrient availability (Kinney et al. 1997, Mansfield et al. 1999, Lindroth et al. 2001b). Third, the birch genotypes used in this study may not accumulate tannins in response to high CO_2. Such differential responses among genotypes have been demonstrated in aspen (*P. tremuloides* Michaux) (Mansfield et al. 1999, Lindroth et al. 2001c).

We found that elevated O_3 reduced nitrogen concentrations and interacted with CO_2 to alter starch concentrations, but did not affect condensed tannin concentrations. The 11% reduction in nitrogen levels is likely due to O_3 affecting the synthesis of Rubisco (Pell et al. 1994, Bortier et al. 2000). Starch levels in the O_3 treatment did not differ from those of trees in the ambient treatment. Our results contrast with those of other studies, where O_3-exposure decreased foliar starch concentrations (Bücker and Ballach 1992, Friend and Tomlinson 1992, Lavola et al. 1994). Such decreases have been attributed to the conversion of starch into soluble sugars used to repair O_3 injury (Lavola et al. 1994). A possible difference in the response of starch between this and the aforementioned studies is that O_3 damage in this study may not have been severe enough to elicit a reduction in starch concentrations. With respect to tannins, our results were similar to another study that found that O_3-exposure did not alter concentrations of condensed tannins, and most simple phenolics, in the birch *Betula pendula* Roth (Lavola et al. 1994). Other studies, however, conducted mostly with conifers, typically documented increases in tannins and related phenolics in response to O_3 (Koricheva et al. 1998).

The elevated $CO_2 + O_3$ treatment resulted in the poorest foliar quality (relatively low nitrogen and high starch and condensed tannin concentrations of all the fumigation treatments). The enhanced reduction in nitrogen is likely the result of multiple factors, including dilution due to an increase in carbon-based metabolites and CO_2- and O_3-mediated reduction in Rubisco concentrations as suggested by Kull et al. (1996).

Insect performance was not significantly altered by the marginal CO_2- or O_3-mediated changes in foliar quality. However, females reared on elevated $CO_2 + O_3$ foliage tended to have smaller pupal masses than insects reared on control foliage. These results are likely due to the combined effect of reduced nitrogen (−27%) and increased tannin (+35%) concentrations. Both changes have been shown to negatively impact insect performance (e.g., Scriber and Slansky 1981, Kopper et al. 2001). The relative difference between male and female responses to elevated $CO_2 + O_3$ foliage may reflect an increased demand for nitrogen (protein) by females to support larger pupal mass and, ultimately, egg production (Lindroth et al. 1997).

The absence of a pronounced CO_2 effect on insect performance was not surprising given the modest change in foliar quality. Other studies have typically shown an increase in development time and/or a decrease in pupal mass for leaf-chewing insects when reared on foliage exposed to elevated CO_2 (Fajer et al. 1991, Lindroth et al. 1993) but there are exceptions (Lindroth et al. 1993, Williams et al. 1997). White-marked tussock moth performance was demonstrably affected by CO_2-mediated changes in birch foliage in previous studies that employed higher CO_2 concentrations ($\approx$700 μl L^{-1}) (Agrell et al. 2000, B.J.K. and R.L.L., unpublished data). Larvae may accommodate minor alterations in foliar chemistry by increasing consumption rates, as has been shown for other herbivores on paper birch (Lindroth et al. 1995).

Regarding larvae reared on O_3-exposed foliage, our results are consistent with the only other long-term study that investigated leaf-chewing insect performance on O_3-exposed trees. Coleman and Jones (1988) found no difference in development time and pupal mass for willow leaf beetles reared on O_3-exposed cottonwood foliage. They attributed the lack of an O_3 effect to increased consumption of ozonated foliage, which allowed beetles to maintain development rates and pupal mass similar to beetles reared on control foliage. Similarly, the lack of a detectable O_3 effect on insect performance in this study could have been masked by increased consumption rates.

Because this is the first study to evaluate the effects of CO_2 and O_3 on foliar quality and insect performance, identification of general patterns is not possible. Our results suggest that some plant-insect asso-

ciations will not be significantly altered under impending atmospheric conditions. Clearly, this does not mean that other insects will not be affected. Future studies should evaluate both additional feeding guilds, and additional measurements of insect performance, such as food consumption, survivorship, and fecundity.

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