Effects of Elevated CO_2 Leaf Diets on Gypsy Moth (Lepidoptera: Lymantriidae) Respiration Rates

ANITA R. FOSS,[1,2] WILLIAM J. MATTSON,[1] AND TERRY M. TRIER[3]

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ABSTRACT Elevated levels of CO_2 affect plant growth and leaf chemistry, which in turn can alter host plant suitability for insect herbivores. We examined the suitability of foliage from trees grown from seedlings since 1997 at Aspen FACE as diet for the gypsy moth (*Lymantria dispar* L.) Lepidoptera: Lymantriidae: paper birch (*Betula papyrifera* Marshall) in 2004–2005, and trembling aspen (*Populus tremuloides* Michaux) in 2006–2007, and measured consequent effects on larval respiration. Leaves were collected for diet and leaf chemistry (nutritional and secondary compound proxies) from trees grown under ambient (average 380 ppm) and elevated CO_2 (average 560 ppm) conditions. Elevated CO_2 did not significantly alter birch or aspen leaf chemistry compared with ambient levels with the exception that birch percent carbon in 2004 and aspen moisture content in 2006 were significantly lowered. Respiration rates were significantly higher (15–59%) for larvae reared on birch grown under elevated CO_2 compared with ambient conditions, but were not different on two aspen clones, until larvae reached the fifth instar, when those consuming elevated CO_2 leaves on clone 271 had lower (26%) respiration rates, and those consuming elevated CO_2 leaves on clone 216 had higher (36%) respiration rates. However, elevated CO_2 had no apparent effect on the respiration rates of pupae derived from larvae fed either birch or aspen leaves. Higher respiration rates for larvae fed diets grown under ambient or elevated CO_2 demonstrates their lower efficiency of converting chemical energy of digested food stuffs extracted from such leaves into their biosynthetic processes.

KEY WORDS gypsy moth, paper birch, trembling aspen, elevated CO_2 effects, insect metabolic rate

Rising CO_2 levels and associated climate change effects on forests have been of concern for decades (Bazzaz et al. 1990, Luo et al. 1999, Norby et al. 1999, Karnosky 2002). Under controlled environments, elevated CO_2 increases biomass production (Bazzaz et al. 1990, Kubiske et al. 2006) and modifies carbon allocation and partitioning in trembling aspen and paper birch (Dickson et al. 2000, Mattson et al. 2005, Kubiske et al. 2006). Such CO_2 induced alterations in leaf and woody tissue physiology and chemistry can have a cascading effect on interactions with phytophagous insects (Hillstrom et al. 2010; Couture et al. 2012; Lindroth 2010, 2012) and pathogens (Percy et al. 2002, Klopfenstein et al. 2009).

In a comprehensive meta-analysis of elevated CO_2 effects on plant-arthropod interactions, Robinson et al. (2012) found general agreement that elevated CO_2 lowers host plant quality for herbivores by decreasing levels of leaf nitrogen and increasing secondary metabolites, but they caution that 'plant quality' is relative to the herbivore in question, owing to its unique sensitivities and nutritional needs. Early studies at Aspen FACE in northern Wisconsin found that leaves of young trembling aspen and paper birch grown under elevated CO_2 generally, but not always, had lower levels of nitrogen and higher levels of starches, fiber, and secondary compounds such as condensed tannins and various species of phenolics than leaves from ambient grown trees (Kinney et al. 1997, Roth et al. 1998, Lindroth et al. 2001). It is axiomatic that the quality and quantity of food consumed by an insect determines its performance in the larval stage, and thus lowered host plant quality often translates into reduced larval weight gain, prolonged stadium duration (Sheppard and Friedman 1990, Lincoln et al. 1993, Lindroth and Kinney 1998, Corviella and Trumble 1999, Agrell et al. 2000, Lindroth et al. 2002, Valkama et al. 2007), and ultimately decreased survival and fecundity (Awmack and Leather 2002).

Standard methods for evaluating food quality of insect diets involve quantifying food consumption and insect growth gravimetrically in a mass and or energy budget and calculating food consumption and utilization efficiencies (Waldbauer 1968). Such indices have been widely used to compare diets, but have some limitations such as (a) compounding magnification of gravimetric errors in measurements, and problems associated with excess uneaten food (Schmidt and Reese 1986, van Loon 1993); (b) potential errors associated with the statistical analysis of ratio data (Raubenheimer and Simpson 1992, Raubenheimer

[1] USDA Forest Service, Northern Research Station, Institute of Applied Ecosystem Studies, 5985 Highway K, Rhinelander WI 54501.
[2] Corresponding author, e-mail: afoss@fs.fed.us.
[3] Department of Biology, Grand Valley State University, 232 Padnos Hall, Allendale, MI 49401.

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1995); (c) controlling for variable larval gut contents (Bowers et al. 1991); and (d) differences between the gravimetric and caloric measurement of food stuffs and insect growth products (Slansky 1985).

Another approach to estimating food quality, particularly its utilization efficiency after passing through the gut wall, is to measure insect metabolism directly using respirometry, or gas exchange (Chown and Nicolson 2004). In measuring the exchange of O_2 and CO_2 per unit time per unit mass of an organism under controlled conditions of temperature, activity, and life stage (Chown and Nicolson 2004), respirometry can measure diet treatment effects on digestibility efficiencies and energy budgets (Lighton and Bartholemew 1988, Chown and Nicolson 2004, Hill et al. 2004, Karasov and Martinez del Rio 2007, Lighton 2008). Respirometry has been useful in understanding patterns of insect metabolism (Gouveia et al. 2000, Fjelden et al. 2001) in relation to environmental factors such as temperature (Bauman et al. 1978, Bennett et al. 1999, Petz et al. 2004), and physiological status such as life stage and diapause (Wagner and Villavaso 1999, Kemp et al. 2004, Canzano et al. 2006). However, respirometry studies of the effects of natural and stress-altered leaf diets on metabolism are less common (Karasov and Martinez del Rio 2007), but can directly estimate the relative efficiency or inefficiency with which an organism processes units of digested food into growth (known as ECD) (van Loon 1993, Bennett et al. 1999), under controlled conditions of temperature, activity, and life stage.

ECD is the standard gravimetric index that reflects metabolic efficiency, but it suffers from the aforementioned problems of all gravimetric nutritional indices. Additionally, because it is purely gravimetric, it cannot delineate between food stored in the body as lipid, protein, or carbohydrate. Because lipid has twice the energy density of protein or carbohydrate, ECD measurements of metabolic efficiency are subject to significantly greater error than respiratory metabolic measurements that are stoichiometrically linked to energy burned in the body for maintainence, growth, detoxication, and locomotion. Slanksy (1985) compared gravimetric versus energy-based studies and found that gravimetric ECDs can be 30% lower than similar energy-based calculations, a result that could be attributed to significant insect growth because of lipid storage. Additionally, respiratory gas methodology can be used to measure metabolic heat production in the acute time frame (second by second in flow-through systems) (van Loon 2005), an advantage that has made respirometery an indispensable and critical investigative tool for over 50 yr in studying postprandial metabolism in animals and quantifying Specific Dynamic Action (SDA), that is, the decline in metabolic efficiency in response to food consumption (McCue 2006, Secor 2009).

Because of the enormous variation in the quality of plant food, insects use flexible feeding behaviors and nutrient utilization strategies to obtain their basic nutritional requirements (Scriber and Slansky 1981, Simpson et al. 1995, Raubenheimer and Simpson 2003). Compensatory feeding, for instance, allows insects to consume in proportion to the concentration of key limiting dietary nutrients, such as nitrogen (Mattson 1980), and they may select alternative foods to supplement a limiting nutrient (Stockhoff 1992, Stoyenoff et al. 1994, Liebhold et al. 1995, Shields et al. 2003), as well as increase digestive sequestration efficiencies (Slansky and Wheeler 1989, Lazarevic et al. 2002). Insects feeding on nitrogen-diluted diets, such as can occur under high atmospheric CO_2 levels, may have to metabolically process more carbohydrates (starches and sugars), fiber, and secondary compounds to sequester all of the essential nutrients required to sustain growth (Zanotto et al. 1997), thereby leading to elevated respiration because of diet induced thermogenesis (Trier and Mattson 2008) and detoxication of allelochemicals, as well as elevated stores of fats (Warbrick–Smith et al. 2006, Trier and Mattson 2008).

In this study, we hypothesized that gypsy moth larvae consuming trembling aspen or paper birch leaves grown under elevated CO_2 would have higher metabolic rates of carbon dioxide production as a result of diminished leaf quality resulting from nutrient dilution, and imbalances, and higher levels of secondary compound toxins, deterrents, or both. Our primary objective was to measure gypsy moth metabolic responses to CO_2 altered tree foliage, and secondarily to measure key leaf traits that are proxies for food quality.

Materials and Methods

Leaf Diet and Leaf Chemistry. Leaves for diet and leaf chemistry were collected from trembling aspen and paper birch trees grown under ambient (380 ppm) or elevated CO_2 (560 ppm) conditions at the Aspen FACE (Aspen Free-Air CO_2 and O_3 Enrichment Project) site, located 15 miles west of Rhinelander, WI (89.5 ° W, 45.7 ° N). Birch trees were established from northern Michigan open-pollinated seed sources and the five aspen clones were propagated from cuttings of trees with previously determined responses to CO_2 and O_3 and planted in a replicated treatment design of ambient CO_2, elevated CO_2, elevated O_3, and combined elevated CO_2 and O_3 (Dickson et al. 2000). Our experiments were conducted with birch in 2004–2005 and aspen clone 271 in 2006 and 216 in 2007, these clones having demonstrated good growth and competitive ability (Kubiske et al. 2006) within the mixed communities at Aspen FACE.

Approximately 5–10 newly matured upper canopy leaves were collected from each of two to five trees accessed from the scaffolding structure in a single ambient and elevated CO_2 experimental ring. Collections were made repeatedly from the same trees in a year (trees varied year to year) every 4–7 d, June–September (to October in 2004), between 0900 and 1200 hours. Leaves were individually handpicked or cut off with pruners, with petioles intact. Leaves from all trees within a treatment ring were combined in a plastic self-sealing bag, transported from the field site

in an ice chest and stored at 4°C for insect feeding. Two leaves per tree from each collection date were kept separate for leaf chemistry analyses. These were processed by removing petioles and taking fresh weights to 0.1 mg (model AT 261, Mettler–Toledo, Columbus, OH) before they were oven-dried for 24 h at 36°C. Dry weights were then taken from which percent water content was calculated ([fresh weight-dry weight/fresh weight] × 100). The oven-dried leaves were ground to 40 mesh in a Wiley Mill and stored in 20 ml plastic scintillation vials. The ground sample was redried for 24 h at 52°C before subsamples of 7–11 mg for carbon-nitrogen analyses and 50–75 mg for condensed tannins-total phenolics analyses were weighed. The carbon and nitrogen percent per dry weight of leaf sample was determined by a high temperature combustion analyzer (FLASH EA1112, CE Elantech, Inc., Lakewood, NJ) that was standardized and calibrated with acetanilide. The carbon to nitrogen ratio (C/N) was calculated as percent carbon divided by percent nitrogen. A methanol-water (1:1 vol:vol) sample extraction was used to determine condensed tannins by the sulfuric acid method and total phenolics by a modification of the Folin–Dennis assay (Bae et al. 1993, Nitao et al. 2001) using continuous rapid flow analysis (RFA-300, Astoria-Pacific, Inc., Clackamas, OR). Purifications of leaves from locally grown birch and aspen trees were prepared as reference standards according to Hagerman and Butler (1980) and for calibrating the rapid flow analyzer.

Insect Rearing. Native gypsy moth egg masses were collected from Boot Lake Campground (Nicolet–Chequamegon National Forest, Langlade Co., WI) for our first experiments in 2004 but the subsequent experiments were conducted with New Jersey (NJ) laboratory strain egg masses that were obtained from the U.S. Department of Agriculture–Aphis Plant Protection and Quarantine Laboratory (Otis ANGB, MA). We followed established procedures for egg mass storage at 4°C and processing and adapted egg sterilization protocols (Odell et al. 1984) using a 10% Formalin (Mallinkrodt–Baker, Phillipsburg, NJ) soak for 2 min and two 2 min sterile H_2O rinses.

Sterilized eggs from a single egg mass were divided into 2–4 cohort groups of ≈50 eggs each. The eggs were placed in polystyrene weigh boats (41 by 41 by 8 mm) set on moistened filter paper covering the bottom of petri dishes (10 by 100 mm). Petri dishes were sealed with Parafilm to maintain moisture and prevent neonates from escaping. Egg incubation and rearing took place in an upright growth chamber (model 307C, Fisher, Pittsburgh, PA) at 25°C and 50% rH with a photoperiod of 16:8 (L:D) h. New insect groups were started approximately every 6 wk between June and September. Two groups of native WI strain insects were reared to pupation and a third group of NJ strain insects to fifth instar on birch diets in 2004. Two additional groups were reared on birch diets to fourth instar in 2005. Four insect groups were reared on aspen clone 271 diets in 2006, three to fifth instar and one to first instar, and two groups were reared to pupation on aspen clone 216 diets in 2007.

Neonates were placed on leaves in petri dishes within 1–2 d of eclosion with treatment assignments to ambient or elevated CO_2 leaf diets made by simple alternate selection and placement. Leaves were kept turgid by submerging leaf petioles into chunks of water-saturated horticultural oasis (1 by 2 by 1 cm). Transfers to fresh leaves in new petri dishes or pint-sized clear plastic containers for larger instars were made every 2–5 d to provide clean rearing conditions and fresh diet.

Insect Respiration. Larval and pupal respiration rates (carbon dioxide production and oxygen uptake) were recorded with a constant volume respirometry system set up in push mode according to Lighton (2008) in which compressed gas regulated to five psi flowed through dessicant and CO_2 scrubber columns before being pumped at controlled flow rates of 100–200 ml/min for early instars and 300–500 ml/min for later instars to the insect chambers and through the gas analyzers. Recording lengths and sequences as well as output normalization were defined within acquisition software (DATACAN in 2004–2005 and EXPEDATA in 2006–2007, Sable Systems, Inc. 2005). Voltage output of the carbon dioxide analyzer (model LI-6251, LICOR, Lincoln, NE) was converted to parts per million by a third degree polynomial and that of the oxygen analyzer (FC-1, Sable Systems, Inc.) to percent oxygen by a linear equation. Gas analyzers were calibrated before recordings. High purity nitrogen was used to zero the Li-6251 and 983.4 ppm carbon dioxide in air (Airgas Inc., Chicago, IL) used for span calibration. The FC-1 was zeroed and span calibrated with compressed dry air of 20.9–21.5% oxygen (Airgas Inc.). A continuous supply of compressed dry air flushed insect chambers and maintained zero baselines between recordings.

Recordings were paired, the first one flushing the insect chambers of residual gases and the second one recording actual gas exchange. Intervals between paired recordings, 15–20 min for early instars and 1–10 min for later instars, provided time for insect recovery and gas exchange. Modifications to the recording program in 2006 shortened recording duration and eliminated paired recordings to bring carbon dioxide output of late instar larvae and pupae within calibration range. Insects were randomly selected from their diet groups and transferred to individual respirometry chambers, 20 by 60 mm for early instars and 20 by 80 mm for later instars. The chambers were inside a 22°C peltier temperature controlled chest. Small foam test tube plugs (with cutout sides) put inside the respirometry chambers served as a resting place for early instar larvae that kept them relatively stationary. Later instar larvae were kept relatively inactive by chamber size. Fresh weights of larvae and pupae were taken after respirometry recordings as body mass covariate to respiration rates. Replicate recordings of insect cohorts at each instar from each diet group were made and data combined for analyses.

Respirometry data were corrected for baseline drift and converted by mathematical operations before analyses. Carbon dioxide data from DATACAN re-

cordings were converted to percent (1 by 10^4) and oxygen data to a proportional fraction (1 by 100^{-1}) for automated analyses in the constant volume utility, CONVOL (Sable Systems, Inc.) with output in milliliters per hour. Carbon dioxide data from EXPEDATA recordings were converted to a fractional concentration (1 by 10^6) and oxygen data to a proportional fraction [1 by 100^{-1}], with additional oxygen signal noise and lag corrections made. Data were further transformed by respirometry formulae for manual integrations by the AREA function in the Analysis Menu, with outputs in milliliter rates of gas that when divided by recording length resulted in milliliters per hour.

Pupal Lipid Extractions. Lipid extractions of pupae from the 2007 aspen clone 216 experiment were done to estimate CO_2 effects on lipid accumulation. Pupae were weighed and then immediately freeze-killed at 2°C. After oven-drying for 72 h at 40°C, preextraction weights were taken to within 0.01 mg (model AT 261, Mettler–Toledo, Columbus, OH). Each pupa was placed in individual wells (Multichem multiplate, Whatman Inc., Florham Park, NJ) containing 3 ml of chloroform with a glass marble on top to keep them submerged. After 8 h, the extractant was removed and replaced with fresh chloroform for a total of four 8 h extractions. Pupae were air-dried then oven-dried 24 h at 40°C before postextraction weights were taken. Lipid content was calculated as the gravimetric difference between pre- and postextraction dry weights $(({}_{\text{pre}}\text{gDwt} - {}_{\text{post}}\text{gDwt})/{}_{\text{pre}}\text{gDwt})$ and proportion lipids as (lipid gDwt/$_{\text{pre}}$ gDwt).

Statistical Methods. Carbon dioxide data from late instar larvae and pupae that exceeded the analyzer's calibration range (>1,500 ppm) and oxygen data from first instar larvae that was indistinguishable from signal noise (<0.0010 ml/min) because of lower end insensitivity of the oxygen analyzer was excluded from analyses. Replicate recordings at each instar from each diet level of CO_2 were combined for analyses as were replicate recordings of cohort groups in the same year. Respirometry and leaf chemistry data from each year were analyzed separately but a combined dataset was created for analyses of clonal effects.

Respirometry response variables (fresh body weight and carbon dioxide ml/h) and leaf chemistry variables (percent moisture, nitrogen, carbon, carbon/nitrogen ratio, condensed tannins, and total phenolics) were assessed for normality and heterogeneity of variances using the Kolmorgorov–Wilks test and Levene's tests, respectively, to determine the best fit distribution (PROC UNIVARIATE, SAS version 9.2, SAS Institute, Cary, NC). Respirometry data and most leaf chemistry data were best fitted by a gamma distribution but a normal distribution was used when appropriate. Data were log transformed for analysis of covariance (ANCOVA). Respirometry and leaf chemistry response variables and proportion pupal lipid per dry weight were analyzed with a generalized linear mixed model suitable for data not normally distributed with heterogeneous variances, PROC GLIMMIX (SAS Institute 2008) (SAS version 9.2), using either a normal or gamma distribution with an identity or log link function, respectively. A repeated measures statement with spatial power covariance structure (SP(POW)) was added to the model for leaf chemistry analyses to account for unevenly spaced leaf collections. A significance value of <0.05 was used for all analyses. The classification factors used in the GLIMMIX or ANCOVA models were diet (High [560 ppm] and Low [380 ppm] CO_2 aspen or birch leaves) for analysis of respiration rates; pupal sex and diet for analysis of proportion lipids in pupae; Julian date and diet for leaf chemistry analyses; and, clone and diet for analyzing fourth and fifth instar weights.

Additionally, we used ANCOVA (PROC MIXED, SAS version 9.2) to determine the relationship of insect mass ($xi1$) to respiration (Yi) with diet (CO_2 level) as the classification factor. The model for the completely randomized ANCOVA is

$$\text{Yi} = \beta 0 + \beta 1\ \text{xi1} + \tau 1\ \text{I(diet = "High")} + \tau 2\ \text{I (diet = "Low")} + \epsilon \text{i} \quad [1]$$

where I (diet = High for elevated CO_2) is the indicator function that returns 1 if the value of the diet variable is "High" and returns 0 if the diet variable is "Low" (diet = Low for ambient CO_2). Parameter $\tau 1$ and $\tau 2$ is associated with the diet classification effect. Link functions appropriate to the distribution of the data further modified the model.

The assumptions for ANCOVA were tested (PROC MIXED, SAS version 9.2) following steps outlined by King (2007) to determine if the relationship between the $\log_e$ transformed CO_2 ml/h response and $\log_e$ transformed fresh weight covariate of the respirometry experiments were linear and significant, if the regression slope coefficients were equal among CO_2 diet levels and if the residuals were normally distributed and homogeneous. Each of the birch experiments, 2004–2005, had equal regression slopes and variances so were analyzed with standard ANCOVA (Table 1). Both of the aspen experiments, clone 271 in 2006 and clone 216 in 2007 had unequal slopes so were analyzed by modifications of the standard ANCOVA to make computations at the median, 10th and 90th percentile covariate locations (Tables 1 and 2). In addition, a repeated statement was added to the model for the 2006 experiment to account for unequal residual variance.

Table 1. Summary of *P* values from analysis of covariance assumptions testing of gypsy moth respiration on High (560 ppm) or Low (380 ppm) CO_2 birch and aspen leaf diets

Year	Species	Instars	N	Slope *P* value	Residual variance *P* value
2004a	Birch	1–5	172	0.9113	0.8434
2004b	Birch	1–5	104	0.8525	0.1216
2005	Birch	1–4	351	0.1754	0.1443
2006	Aspen	1–5	231	**0.0108**	**0.0010**
2007	Aspen	1–5	131	**0.0051**	0.1903

2004a summer birch, 2004b late season birch.

P values <0.05 are bolded to indicate unequal regression slopes and unequal residual variances among treatments.

Table 2. Weight adjusted least square mean (±95% CI) larval respiration rates on High (560 ppm) or Low (380 ppm) CO_2 aspen clone 271 and clone 216 leaf diet from ANCOVA analyses at median, 10%, and 90% location

Clone	CO_2	CO_2 ml/h		
		Median	10%	90%
271	High	0.01984 (0.02116, 0.01859)	0.00269 (0.00287, 0.00252)	0.23444 (0.27309, 0.20126)
	Low	0.02164 (0.02304, 0.02033)	0.00248 (0.00273, 0.00225)	0.31554 (0.36309, 0.27418)
		$P = 0.0564$	$P = 0.1581$	**$P = 0.0050$**
216	High	0.01320 (0.01422, 0.01225)	0.00211 (0.00233, 0.00192)	0.30796 (0.35367, 0.26816)
	Low	0.01263 (0.01355, 0.01177)	0.00235 (0.00264, 0.00210)	0.22690 (0.26412, 0.19493)
		$P = 0.3938$	$P = 0.1574$	**$P = 0.0039$**

P values <0.05 are bolded if means ± 95% CI are significantly different based on Tukey–Kramer grouping.

Results

Julian Date and CO_2 Effects on Leaf Chemistry. As expected, there were seasonal changes in almost all traits in aspen, but surprisingly only moisture and carbon content, and C/N ratio varied seasonally in birch (Table 3; Figs. 1 and 2). Generally, when Julian collection date was significant, it was often because of steady seasonal trends of declining nitrogen, phenolics, moisture, and increasing carbon and C/N ratio. However, there was also evidence of nonlinear, that is, concave changes for phenolics and C/N (Figs. 1 and 2). Contrary to expectation, the CO_2 treatment generally had no detectable effect on any measured chemical trait in aspen or birch, except for carbon content in birch, and moisture content in aspen, where, ambient leaves had higher total carbon and moisture content, respectively (Table 3). With the exception of moisture content in birch, 2004, and condensed tannins in aspen, 2007, there were no significant interactions between CO_2 and Julian date.

CO_2 Effects on Larval Respiration. The CO_2 exposure levels of ingested leaves had a significant effect on

Table 3. Summary of *F* and *P* values for the effects of CO_2 (High, 560 ppm or Low, 380 ppm), Julian date and their interactions on birch and aspen leaf chemistry

Main effects and interactions		Moisture	Nitrogen	Carbon	C/N	Tannins	Phenolics
Birch 2004 CO_2							
df	*F*	0.00	0.00	6.08	0.02	1.02	1.35
1, 13	*P*	0.9599	0.9967	**0.0283**	0.8964	0.3314	0.2668
Jdate							
df	*F*	8.89	1.83	0.98	2.10	0.48	1.06
11, 77	*P*	**<0.0001**	0.0638	0.4722	**0.0300**	0.9125	0.4015
CO_2 × Jdate							
df	*F*	2.38	0.42	1.31	0.19	1.20	1.80
11, 77	*P*	**0.0134**	0.9413	0.2369	0.9976	0.2992	0.0677
Birch 2005 CO_2							
df	*F*	2.64	0.47	4.50	0.11	0.08	2.00
1, 8	*P*	0.1427	0.5112	0.0666	0.7520	0.7860	0.1951
Jdate							
df	*F*	3.22	1.53	7.40	0.87	1.10	0.25
5, 30	*P*	**0.0191**	0.2112	**0.0001**	0.5118	0.3810	0.9377
CO_2 × Jdate							
df	*F*	0.71	1.64	2.26	2.28	0.25	1.37
5, 30	*P*	0.6226	0.1808	0.0738	0.0721	0.9343	0.2631
Aspen 2006 CO_2							
df	*F*	27.94	0.01	0.41	0.01	0.36	0.09
1, 6	*P*	**0.0019**	0.9373	0.5443	0.9367	0.5719	0.7760
Jdate							
df	*F*	10.0	3.81	2.87	4.07	1.18	2.63
14, 37	*P*	**0.0001**	**0.0006**	**0.0052**	**0.0003**	0.3287	**0.0095**
CO_2 × Jdate							
df	*F*	1.40	0.78	0.91	0.72	1.58	1.52
14, 37	*P*	0.1997	0.6879	0.5552	0.7402	0.1303	0.1509
Aspen 2007 CO_2							
df	*F*	3.99	0.02	2.88	0.72	0.32	0.18
1,4	*P*	0.1165	0.8904	0.1648	0.4427	0.6029	0.6929
Jdate							
d.f.	*F*	1.98	5.68	4.53	3.34	3.21	3.77
10, 27	*P*	0.0771	**0.0001**	**0.0008**	**0.0060**	**0.0076**	**0.0029**
CO_2 × Jdate							
df	*F*	1.02	0.71	0.83	0.47	2.35	1.37
10, 27	*P*	0.4527	0.7071	0.6084	0.8922	**0.0378**	0.2481

P values <0.05 are bolded; C/N carbon to nitrogen ratio.

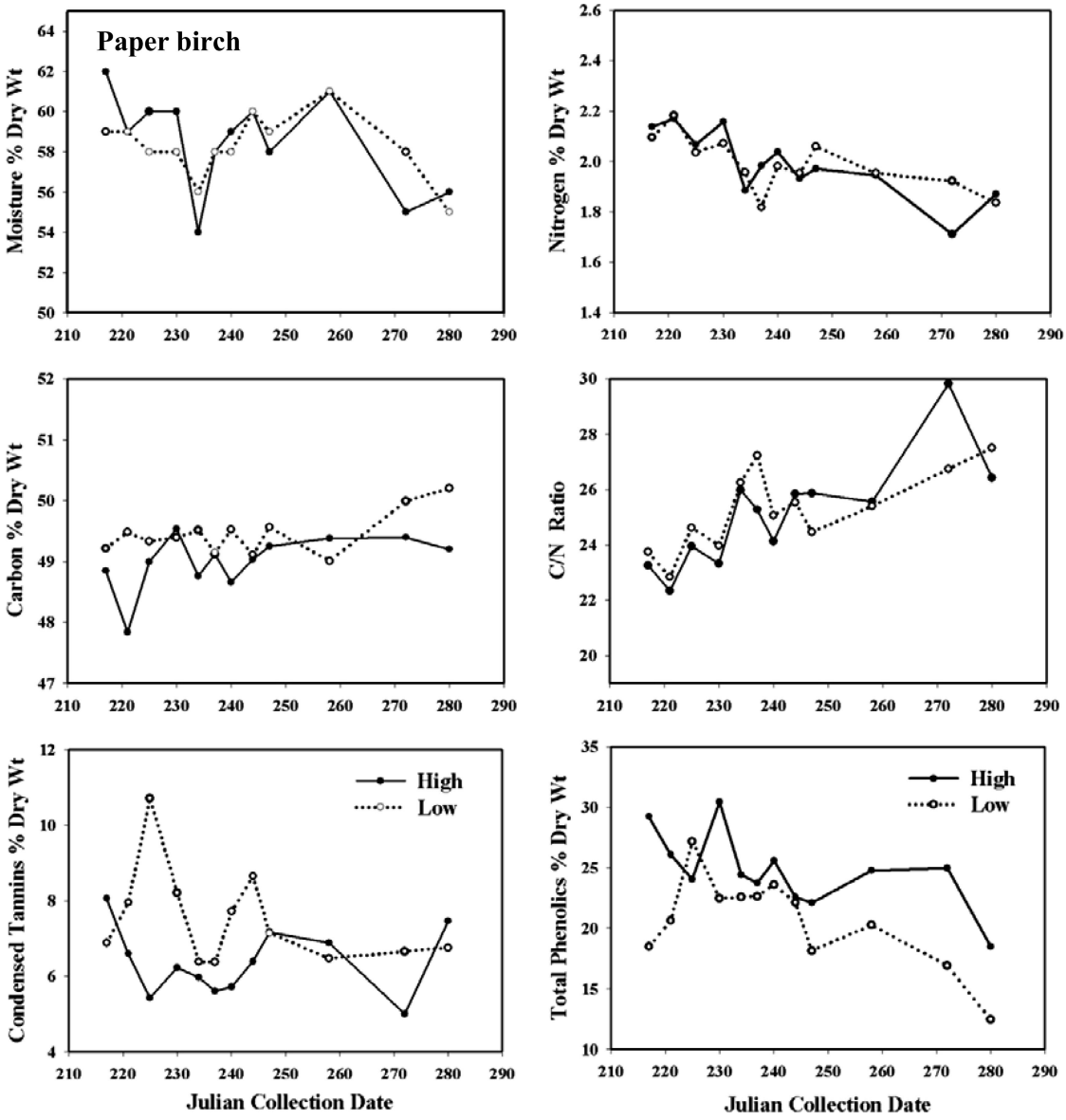

Fig. 1. Effects of High (560 ppm) and Low (380 ppm) CO_2 levels on paper birch leaf chemistry (moisture, nitrogen and carbon, C/N ratio, condensed tannins, and total phenolics), for Julian dates 210–290 (Aug.–Oct.) in 2004.

gypsy moth larval respiration rates in four of five experiments. For example, feeding on elevated CO_2 birch leaves increased larval respiration rates from 15 to 59% in two of three experiments (2004a and 2005), but had no significant effect in the October 2004b experiment (Table 4). Conversely, the opposite was true for aspen where feeding on elevated CO_2 leaves decreased larval respiration rates ≈25–45%, although the effect was significant only in 2006, but not in 2007 (Table 4).

Exploring CO_2 effects in greater detail, using regression plots, and comparing the slopes and variances of ambient and elevated CO_2 regressions lines ($\log_e$ respiration rate versus $\log_e$ body fresh weight), we learned that in each of the three birch trials the regression slopes were equal and variances homogeneous for ambient and elevated CO_2 cohorts. Elevated CO_2 treatments resulted in significantly higher gypsy moth respiration rates per unit mass in the 2004a and 2005 experiments, but not in the 2004b one, as regression lines reveal (Fig. 3), agreeing fully with the above GLIMMIX analyses. However, for aspen experiments, ambient and elevated CO_2 cohorts had unequal regression slopes in both 2006 (clone 271) and 2007 (clone 216) and heterogeneous variances in 2006 (Table 1). Therefore, we calculated the regression line divergences for the 2006 and 2007 data at the median, 10%, and 90% body mass covariate. The tests revealed that elevated CO_2 in 2006 decreased respiration rates

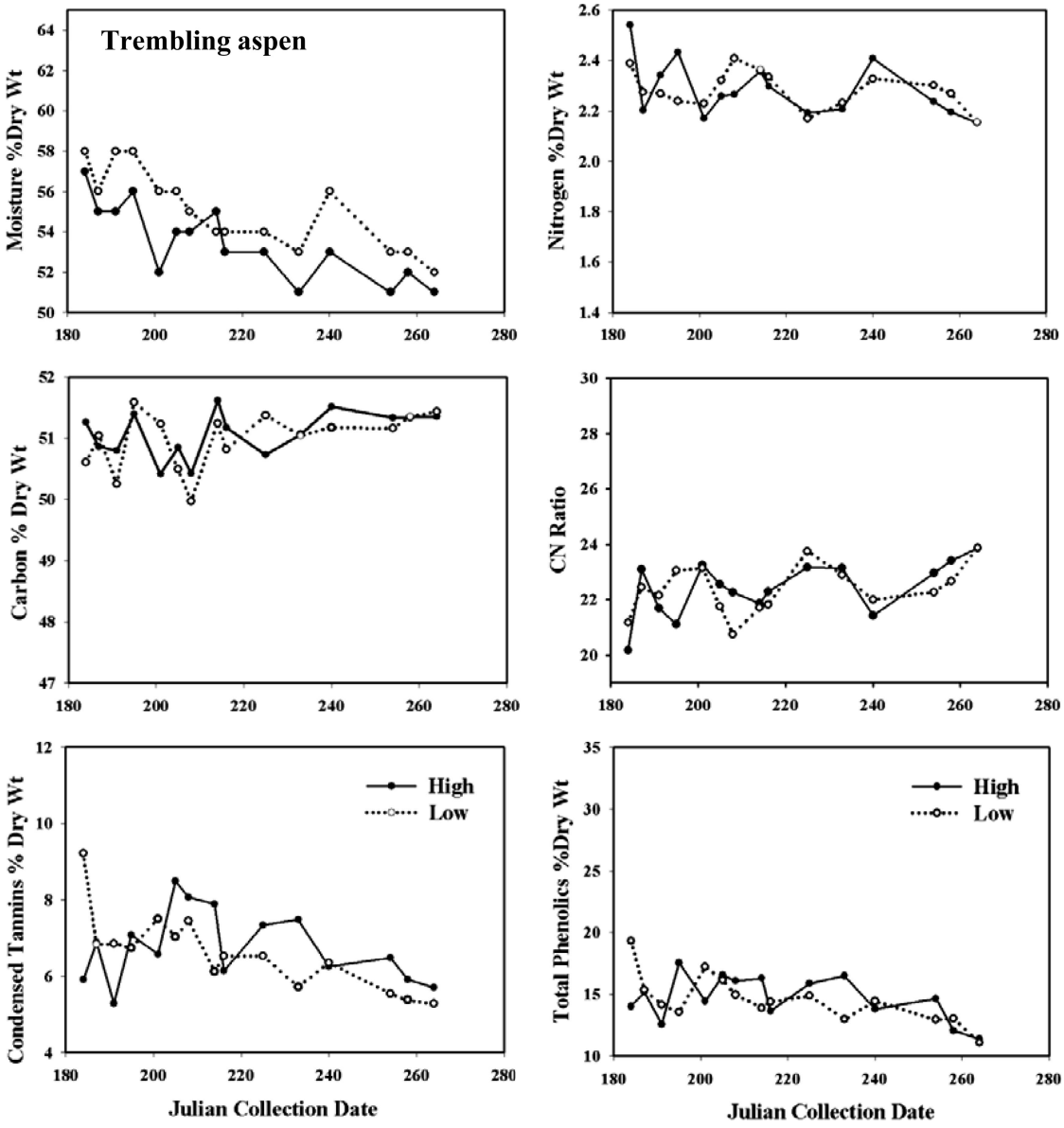

Fig. 2. Effects of High (560 ppm) and Low (380 ppm) CO_2 levels on trembling aspen leaf chemistry (moisture, nitrogen and carbon, C/N ratio, condensed tannins, and total phenolics), for Julian dates 180–280 (July–Sept.) in 2006.

25.7% at larger body size, but not at median and smaller body sizes (Table 2), whereas in 2007, elevated CO_2 increased respiration rates by 35.7% at larger body sizes, but not at median and smaller sizes. Therefore, these more detailed analyses revealed specifically that the elevated CO_2 treatments decreased larval respiration rates on clone 271, and increased respiration rates on clone 216, but only at the larger body sizes, as is evident from the diverging regressions lines (Fig. 3).

CO_2 Effects on Pupal Respiration and Lipid Content. Elevated CO_2 treatments had no significant effect on the respiration rates of pupae derived from larvae feeding on birch in the 2004a experiment or those from the aspen clone 216 experiment in 2007 (Table 4). However, there was a significant CO_2 effect ($P = 0.045$) on dry weight lipid accumulation in pupae from the aspen clone 216 experiment, with pupae from elevated CO_2 diet having lower lipid content (36.2%) than those from ambient diet (45.2%) (Table 5).

Discussion

The early phytochemistry studies at Aspen FACE (on seedling and saplings) suggested that birch may be more responsive than aspen to elevated CO_2. More consistent differences were found in the many and various classes of chemical traits of birch foliage and woody tissues (e.g., secondary compounds, sugars,

Table 4. Weight adjusted least square mean (± SEM, *N*) respiration rates of gypsy moth larvae and pupae from High (560 ppm) or Low (380 ppm) CO_2 aspen and birch diets

Year	Species	Instars	CO_2	CO_2 ml/h
2004a	Birch	1–5	High	**0.02766 (±0.0023, 79)**
			Low	0.01895 (±0.0015, 94)
2004b	Birch	1–5	High	0.02517 (±0.0029, 55)
			Low	0.02489 (±0.0030, 51)
2005	Birch	1–4	High	**0.01388 (±0.0005, 180)**
			Low	0.01173 (±0.0005, 173)
2006	Aspen	1–5	High	0.01221 (±0.0011, 169)
			Low	**0.02058 (±0.0016, 211)**
2007	Aspen	1–5	High	0.01875 (±0.0022, 63)
			Low	0.02459 (±0.0027, 70)
2004a	Birch	pupae	High	0.07790 (±0.0034, 30)
			Low	0.06850 (±0.0034, 23)
2007	Aspen	pupae	High	0.20930 (±0.0193, 24)
			Low	0.18730 (±0.0189, 20)

2004a summer birch, 2004b late season birch.

LS Means (± SEM, *N*) are bolded to indicate Tukey–Kramer separation of significant CO_2 level effects at $P < 0.05$ in GLIMMIX analyses (SAS Institute 2008).

Table 5. Summary of *F* and *P* values for the effects of CO_2, sex, and their interaction on proportion lipids per gram pupal dry weight

Main effect and interaction	df	*F* value	*P* value
CO_2	1, 42	4.29	**0.0446**
Sex	1, 42	4.26	**0.0453**
$CO_2 \times$ sex	1, 42	3.07	0.0870

P values <0.05 are bolded. CO_2 levels are High (560 ppm) and Low (380 ppm).

and starches) between ambient and elevated CO_2 treatments, coupled with lower nitrogen under elevated conditions (Mattson et al. 2004, 2005). For example, among the first studies, Lindroth et al. (2001) found that elevated CO_2 increased birch foliar tannins, starches, and C/N ratios, and decreased nitrogen. However, Kopper et al. (2001) found no CO_2 effect on birch leaf chemistry at Aspen FACE. Likewise did Agrell et al. (2005), who reported that elevated CO_2 had no significant effect on birch foliar nitrogen, moisture, starch, and tannins.

Our study methods differ from these earlier studies in that we used only recently matured leaves throughout the entire summer, that is, those that were in full light at the tips of newly growing branch shoots, the kind preferred by gypsy moth and many other free-feeding folivores. Therefore, the leaves in this study

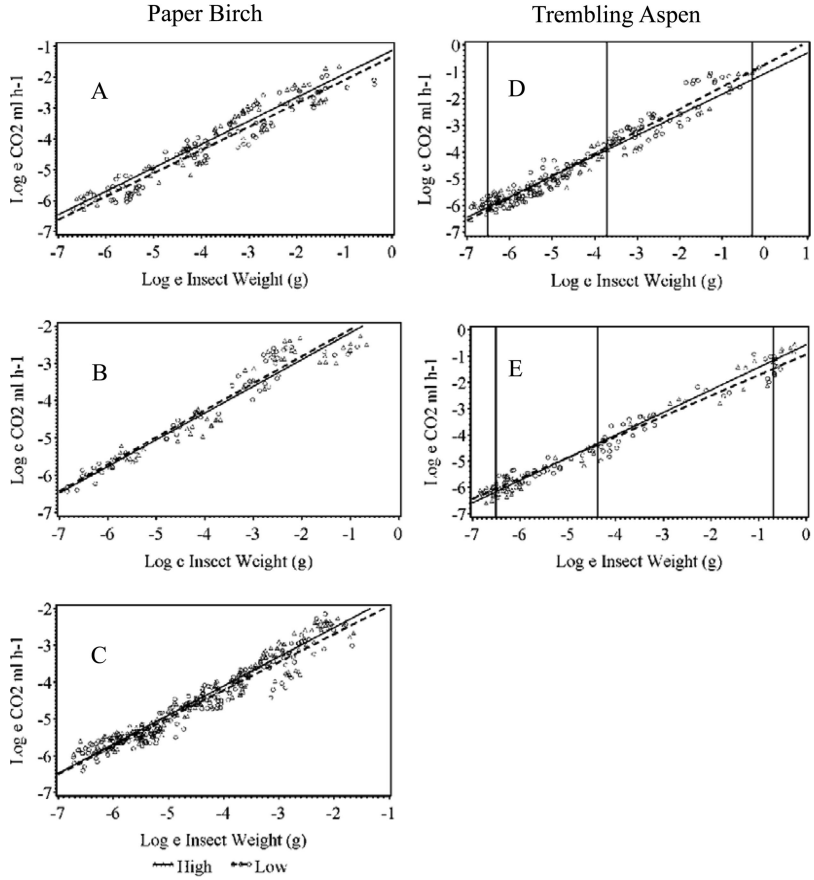

Fig. 3. Bivariate plots of gypsy moth CO_2 ml/h production by gram body weight, grouped by High (560 ppm) and Low (380 ppm) CO_2 effects: paper birch in 2004a (A), 2004b (B), and 2005 (C) and trembling aspen clone 271 in 2006 (D) and 216 in 2007 (E). Lines in Plots D and E indicate analyses at 10%, median, and 90% covariate location.

were generally developmentally younger than in previous studies, making them even more likely to be similar because they had less time to differentiate under the two divergent CO_2 treatments. Secondly, our sample trees had been growing under fumigation for ≈7–9 yr, and were no longer seedlings or saplings, but trees 6+ m tall. Our measurements only tested for the abundance of entire classes of compounds, not individual species in those general classes, as for example, total phenolics and total condensed tannins, each of which may contain several to a dozen species that may respond uniquely to the treatments.

Despite apparent similarities in measured leaf traits between ambient and elevated CO_2 conditions in this study, gypsy moth larvae nevertheless had higher respiration rates on elevated CO_2 birch leaves in the summers of 2004 and 2005. Higher respiration implies that they were less efficient at converting digested elevated CO_2 leaf assimilates into growth. This may be a result of their processing higher loads of diluents such as carbohydrates, nutrient imbalances, and detoxifying or otherwise processing higher loads of some noxious secondary compounds as has been found in other studies (Swain 1977, Marks and Lincoln 1996, Bezemer and Jones 1998, Kopper et al. 2001, Tsao et al. 2002). However, in our October experiment, ambient and elevated CO_2 birch leaves, still fully green, were processed equivalently by gypsy moth larvae, implying no substantial differences in their fundamental primary and secondary phytochemistry, as perceived by the caterpillar's feeding, digestive, detoxication, and metabolic systems. Our October measurements of total phenolics (see Fig. 1) suggest that elevated CO_2 birch leaves were clearly tending toward higher phenolic levels. However, at that time of year, trees are translocating essential nutrients out of their leaves before leaf drop, and perhaps ambient and CO_2 leaves were not markedly different from the insect's perspective. Our study differs from two earlier studies on insect performance on birch at Aspen FACE, both of which have been consistent, in finding no CO_2 effects on the feeding preferences and growth and development of two folivores, the whitemarked tussock moth (*Orgyia leucostigma* J.E. Smith) (Kopper et al. 2001), and the forest tent caterpillar (*Malacosoma disstria* Hübner) (Agrell et al. 2005).

Although we did not find differences in aspen leaf chemistry between elevated and ambient CO_2 treatments, early studies at Aspen FACE found that elevated CO_2 reduced aspen leaf nitrogen levels by 5–21%, and increased C/N ratios, and phenolic glycosides, but had no effect on starches, sugars, and tannins (Lindroth et al. 2001, 2002). In their study, clones 216 and 271 had similar nitrogen, but different tannin levels. In a later study, Kopper and Lindroth (2003) reported that elevated CO_2 reduced Aspen FACE foliar nitrogen levels by 11%, but had no effect on tannins or phenolic glycosides. Similarly, Holton et al. (2003) found that elevated CO_2 decreased nitrogen levels in two Aspen FACE clones, but had no effect on carbon-based secondary compounds, and there were no significant $CO_2 \times$ clone interactions. Agrell et al. (2005) differed from Holton et al. (2003) by finding a $CO_2 \times$ clone interaction where there were no CO_2 effects on clone 216, but caused elevated starch and tannins in clone 259. Most recently at Aspen FACE, Vigue and Lindroth (2010) reported that elevated CO_2 had negligible effect on aspen phytochemistry (except perhaps for tannins, $P = 0.07$). Of all the complexity in aspen phytochemistry, Osier and Lindroth (2004) and Vigue and Lindroth (2010) concluded that differences in phenolic glycosides and condensed tannins levels explain most of the variation in folivore performance.

In our study, gypsy moth respiration rates did not respond to elevated CO_2 treated aspen leaves of either clone until late instar when larvae had 26% lower respiration rates (i.e., higher efficiency of digested food conversion) on clone 271 and 37% higher respiration rates on clone 216. Genotypic differences in phytochemistry among aspen clones are well known (Osier et al. 2000, Vigue and Lindroth 2010) and likely to influence insect respiration differences between these clones. Lipid accumulation also was sensitive to CO_2 treatments of clone 216, because gypsy moth pupae had 22% lower lipid levels on elevated than on ambient CO_2 diets. All of the earlier studies of elevated CO_2 effects on aspen folivores at Aspen FACE have largely found negligible effects. For example, Lindroth et al. (2002) reported no significant CO_2 effect on the growth of whitemarked tussock moths feeding on clones 216 and 271, but shortened development times on 271. Likewise, Kopper and Lindroth (2003) reported no CO_2 effects on the performance of the aspen blotchminer (*Phyllonorycter tremuloidiella* Braun), as did Holton et al. (2003) on the growth and development of the forest tent caterpillar. In addition, Agrell et al. (2005) found no CO_2 effects on feeding preferences of the forest tent caterpillar on clone 216, but significant nonpreference on clone 259. In the last folivore study at Aspen FACE, Vigue and Lindroth (2010) reported no CO_2 effects on any performance measure of the aspen leaf beetle (*Chrysomela crotchi* Brown). Measurements of insect fitness correlate generally but not perfectly with metabolic rate (Karasov and Martinez del Rio 2007) because organisms compensate for variations in diet quality and its digestion by various physiological and behavioral strategies (Hill et al. 2004, Karasov and Martinez del Rio 2007).

In summary, after >10 yr of research at Aspen FACE, we believe that our study has demonstrated more CO_2 effects on a folivore there than any previous study. The evidence suggests that elevated CO_2 diminished foliar food quality for gypsy moth larvae in birch, and aspen clone 216, but enhanced it in aspen clone 271, and this was the case even though we were unable to detect significant CO_2 induced changes in foliar chemistry in either plant species. As reviewed above, the majority of prior studies at Aspen FACE also had difficulty consistently finding CO_2 induced phytochemical changes after the seedling, and small sapling stage. There is a clear need for more research to identify the specific nutrients and phytochemicals and their interactions that respond to elevated CO_2

and to determine whether a particular insect folivore is negatively or positively influenced by such changes. Our research, nevertheless, supports the hypothesis that rising levels of CO_2 can indirectly influence herbivores and may have broad reaching impacts (Stiling et al. 2003; Hall et al. 2005; Robinson et al. 2010; Lindroth 2010, 2012) that are still poorly appreciated and understood. Moreover, the nature and direction of interactions between plant and herbivore species is highly complex, varying not only by species, but also by genotype, and environment, thwarting simple and sweeping generalizations (Lindroth 2012). Changing climates will likely be highly important in affecting plant-insect interactions.

Using the Pleistocene estimates of CO_2 levels as a guide, we know that plants and herbivores have coevolved while experiencing swings in levels of CO_2 that were bounded between roughly 180 and 280 ppm (Sage and Coleman 2001), seemingly small compared with the imposed 200 ppm difference between Aspen FACE ambient (360–380 ppm) and the elevated (560 ppm) CO_2 treatments. Plants may have passed through an evolutionary bottleneck brought about by highly deficient CO_2 levels during the Pleistocene, and their ensuing deficit adaptations may now constrain their needed phenotypic plasticity to quickly acclimate and evolutionarily adapt to the unprecedented 21st century surfeit of CO_2 (now at 391 ppm, and rapidly rising) and highly influential accompanying temperature and moisture swings, along with a ubiquitous plethora of challenging herbivore species such as pathogens and insects (Sage and Coleman 2001). For example, our data and that of the numerous studies of Lindroth et al. (2001, 2002), as well as others mentioned above, suggest that the tree's secondary compound responses to Aspen FACE CO_2 treatment levels at ≈280 ppm above the geological mean high (280 ppm), and ≈170 ppm above our current ambient and historical high (391 ppm) may not be linear, and could have already reached an asymptote at well below current ambient, determined by evolutionarily constrained plant capacities and their responses to crucial underpinning factors such as local soil, temperature, and moisture conditions. Therefore, the secondary compounds that have antiherbivore properties may already be expressed at their maximum level, and thus show small or negligible responses to further increases in CO_2.

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