# Influence of elevated $CO_2$, nitrogen, and *Pinus elliottii* genotypes on performance of the redheaded pine sawfly, *Neodiprion lecontei*

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**Abstract:** Slash pine (*Pinus elliottii* Engelm. var. *elliottii*) seedlings were grown in open-top chambers receiving ambient or elevated atmospheric $CO_2$ (~365 or ~720 $\mu L \cdot L^{-1}$). Seedlings received low or high soil nitrogen treatments (0.02 or 0.2 mg $N \cdot g^{-1}$) and represented three families varying in resistance to fusiform rust (C*ronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Hedgc. & N. Hunt) Burdsall & G. Snow). Following 18 months of exposure to treatment conditions, current-year needles were fed to larvae of the redheaded pine sawfly (*Neodiprion lecontei* (Fitch)). Needle N concentration and water content were lower in elevated-$CO_2$ and in low-N treatments. Total phenolics increased under high-$CO_2$ and low-N conditions and were highest in the resistant family. Condensed tannins did not vary on the basis of $CO_2$ or N but were higher in needles from the resistant family. Alterations in needle chemistry were associated with variations in sawfly growth and development. Larvae performed most poorly on the family most resistant to fusiform rust, suggesting that the mechanism for resistance was similar in both cases. Relative consumption rates increased with $CO_2$-enriched needle diets but were depressed for resistant needles, suggesting deterrence from the higher total phenolics in this family. Diets using $CO_2$-enriched needles or resistant needles or needles from low-N fertilization treatments resulted in lower relative growth rates for the larvae. Days to pupation increased for larvae fed $CO_2$-enriched and low-N needles. These results suggest that the redheaded pine sawfly could suffer as the level of atmospheric $CO_2$ continues to rise.

**Résumé :** Des semis de pin d'Elliott typique (*Pinus elliottii* Engelm. var. *elliotii*) ont été cultivés en chambres découvertes alimentées en $CO_2$ atmosphérique à pression ambiante ou élevée (~365 ou ~720 $\mu L \cdot L^{-1}$). Les semis, représentant trois familles dont la résistance à la rouille fusiforme, *Cronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Hedgc. & N. Hunt) Burdsall & G. Snow était différente, ont également reçu des traitements de fertilisation faible ou élevée (0,02 ou 0,2 mg $N \cdot g^{-1}$) en azote. Après 18 mois d'exposition aux conditions expérimentales, les aiguilles de l'année courante ont été données à manger à des larves de diprion de LeConte (*Neodiprion lecontei* (Fitch)). La concentration en azote et la teneur en eau des aiguilles étaient plus faibles dans les traitements enrichis en $CO_2$ et pauvres en azote. Ces mêmes conditions ont contribué à l'augmentation des phénols totaux, qui étaient les plus élevés dans la famille résistante. Ni le $CO_2$ ni l'azote n'ont fait varier les tannins condensés qui étaient plus élevés dans la famille résistante. Les modifications de la chimie foliaire étaient associées à des variations dans la croissance et le développement du diprion. La moins bonne performance larvaire a été obtenue en présence de la famille la plus résistante à la rouille fusiforme, ce qui indique que le mécanisme de résistance était similaire dans les deux cas. Le taux relatif d'ingestion était plus élevé avec les aiguilles provenant des traitements enrichis en $CO_2$, mais il était faible avec les aiguilles résistantes, indiquant que les composés phénoliques totaux, en plus grande quantité dans cette famille, avaient un effet répulsif. L'alimentation à base d'aiguilles provenant soit des familles résistantes, des milieux enrichis en $CO_2$ ou des traitements de fertilisation faible en azote s'est traduite chez les larves par des taux de croissance plus faibles. Les larves nourries avec des aiguilles enrichies en $CO_2$ et pauvres en azote ont mis plus de temps pour atteindre la pupaison. Ces résultats indiquent que l'augmentation persistante du $CO_2$ atmosphérique pourrait nuire au diprion de LeConte.

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## Introduction

A doubling of the current atmospheric $CO_2$ level, which has been predicted for this century (Keeling and Whorf 1994), may alter plant characteristics (e.g., morphological, physiological, biochemical), which could in turn affect insect herbivores. For example, carbohydrates often accumulate in $CO_2$-enriched plants (Tognetti et al. 1998), and this might lead to increased production of plant defense compounds, as predicted by the carbon–nutrient balance theory (Bryant et al. 1983). Although the response is not always consistent, a number of studies have reported increases in secondary compounds with antiherbivore roles (e.g., tannins) in elevated $CO_2$ (Pritchard et al. 1997; Peñuelas and Estiarte 1998).

In addition, the increased carbohydrates and accelerated plant growth that can occur under elevated $CO_2$ may cause a dilution of nitrogen (N) levels in plant tissue (Mousseau and Enoch 1989; Johnson and Lincoln 1990). Because N is a limiting nutrient for insects (Mattson 1980), $CO_2$-mediated decreases in N levels could negatively affect herbivore performance (Roth and Lindroth 1994; Lawler et al. 1997). Nitrogen fertilization may, in part, counteract the lower foliar N concentrations caused by high $CO_2$ (Hättenschwiler and Schafellner 1999). Also, soil N fertility can affect plant responsiveness to $CO_2$ levels. Soil N may differ as a result of variation in natural availability and (or) N deposition (Köchy and Wilson 2001). Previous studies have shown that some plant responses to high $CO_2$ may only occur, or are of greater magnitude, if nutrients, such as N, are in sufficient supply (Curtis et al. 1995; Prior et al. 1997).

A plant's genotype can affect how it will respond to elevated $CO_2$, in comparison with other individuals within that species (intraspecific differences). These differences between individual plants under elevated-$CO_2$ conditions could in turn affect herbivores consuming these plants. If one genotype has a lower N concentration or produces a higher concentration of defense compounds under elevated-$CO_2$ conditions than other genotypes, it may be a less nutritious or palatable food source for herbivores. Ultimately, this situation could result in certain plant genotypes being selected over others (Mansfield et al. 1999; Roumet et al. 1999). Thus far, experiments on the effects of elevated $CO_2$ on different genotypes have yielded conflicting results, ranging from tested genotypes responding similarly in a given experiment (Fajer et al. 1992; Johnsen and Major 1998) to differential responses by varying genotypes (Goverde et al. 1999; Pritchard et al. 2000).

Insect reactions to $CO_2$-enriched foliage also show variability, with species-specific responses (Coviella and Trumble 1999) or different developmental instars of the same species even being influenced differently (Williams et al. 1997*a*). Some insects have experienced little change in performance (Arnone et al. 1995) or have even reacted positively to diets of plants from elevated-$CO_2$ environments (Awmack et al. 1997; Goverde et al. 1999). Most studies, however, have reported some detrimental effects on insects feeding on foliage produced under high-$CO_2$ conditions. Increased consumption rates, decreased growth rates and larval mass, prolonged development times, and reduced digestion efficiencies have all been noted (Lincoln and Couvet 1989; Lindroth et al. 1993; Roth and Lindroth 1994).

Our study examined the individual and interacting effects of the following: $CO_2$ levels, soil N availability, and plant genotype of slash pine (*Pinus elliottii* Engelm. var. *elliottii*) on herbivory by larvae of the redheaded pine sawfly (*Neodiprion lecontei* (Fitch)). We studied the genotype aspect by using three full-sib families of slash pine that had differing levels of resistance to fusiform rust (C*ronartium quercuum* (Berk.) Miyabe ex Shirai f. sp. *fusiforme* (Hedgc. & N. Hunt) Burdsall & G. Snow). An additional objective was to examine treatment effects on foliar chemistry to identify mechanisms responsible for effects on insect performance. This study provides a view of *N. lecontei* response to these environmental changes and to plant intraspecific genetic differences from early instar to adult.

### Study species

*Pinus elliottii* var. *elliottii* is generally found in low, wet habitats in the southeastern United States (Preston 1989). It is a fast-growing tree (Rushforth 1987), and its wood is valuable for construction and pulp. The seedlings used in this study had varying levels of resistance to fusiform rust: susceptible, differential, and resistant, according to previous experiments with bulk inoculum of the fungus (Stelzer et al. 1999). Their method of resisting fusiform rust is not known. Furthermore, their defense against *N. lecontei* has not been demonstrated.

*Neodiprion lecontei* (Hymenoptera: Diprionidae) is a major pest of pines in eastern North America. The larvae consume needles and soft bark from young trees less than 6 m in height (Wilson et al. 1992). Sawfly outbreaks can stunt or kill trees if defoliation is severe enough (Wilson et al. 1992).

## Materials and methods

### Seedling history and preparation

Three-inch (1 in. = 2.54 cm) cuttings were made from 4-year-old *P. elliottii* hedges in Saucier, Mississippi, on May 13, 1996. The cuttings from each resistance class (e.g., all the susceptible cuttings) were full-sibs of each other, having the same parental background. The cuttings were products of the following crosses: resistant, 18-27 × 9-2; differential, 8-7 × 18-26; and susceptible, 18-26 × 18-61 (Stelzer et al. 1999; H.E. Stelzer, unpublished data). After being shipped overnight, the cuttings were rooted at the propagation facility of Alabama A&M University, Huntsville, Alabama, in May 1996. Needles were removed from the basal inch of the cutting, and the basal end was dipped into a modified Hare's rooting compound. Cuttings were then set in 25-$cm^3$ Ray Leach Super Cells (Stuewe & Sons Inc., Corvallis, Ore.), filled with a mixture of peat–perlite (1:3 *v*/*v*). The plants received intermittent mist by 26.5 $L \cdot h^{-1}$ Ray-Jet mist nozzles for 90 days (intervals: 0700–1200, turned on for 8 s every 20 min; 1200–2000, 8 s every 15 min; 2000–0700, mist system off). Greenhouse temperatures were 26.7 °C (day) and 21.1 °C (night). Root rot pathogens were controlled with Chipco 26019 (Rhône-Poulenc Ag Co., Research Triangle Park, N.C.) and Banrot fungicides (Scotts-Sierra Horticultural Products Co., Marysville, Ohio), rotated on a weekly basis. Following the initial

90-day period, cuttings were hardened off for 3 weeks by progressively increasing the interval between mist cycles. The plants received weekly applications of Peter's 20:20:20, $N–P_2O_5–K_2O$ (100 ppm N) and two applications of Sprint 330 (chelated Fe; Novartis Crop Protection, Basel, Switzerland). Cuttings were moved to the Auburn University, Auburn, Alabama, greenhouse in October 1996 and maintained there until January 1997. At that time, cuttings were transplanted into 3.78-L tree containers ("Tall one", 10.2 cm × 35.6 cm, Stuewe & Sons Inc., Corvallis, Ore.) containing vermiculite, peat, and perlite (2:2:1 by volume).

In January 1997, the potted seedlings were set in open-top chambers with rain-exclusion caps (for description, see Mitchell et al. 1995) designed to deliver either ambient- or elevated-$CO_2$ concentrations (~365 or ~720 $\mu L \cdot L^{-1}$). The study site was an outdoor soil bin facility at the USDA–ARS National Soil Dynamics Laboratory in Auburn, Alabama. There were six blocks, each with 1 ambient and 1 elevated-$CO_2$ chamber (randomly assigned), for a total of 12 chambers. There was one tree for each of the family × N treatment combinations (e.g., resistant–low N), for a total of six trees in each chamber. The N treatments were modified from Bazzaz and Miao (1993) and consisted of 0.02 (low N) or 0.2 (high N) mg $N \cdot g^{-1}$. Nitrogen treatments, in the form of sulphur-coated urea (38:0:0, N–P–K), were applied at planting and every 3 months thereafter. Other nutrients were supplied, at nonlimiting levels, in the form of sulphur-coated potassium (0:0:47, N–P–K; 0.04 mg $K \cdot (g\ soil^{-1}) \cdot year^{-1}$) and Micromax Plus (0:4:0, $N–P_2O_5–K_2O$; P = 0.14, Ca = 0.57, Mg = 0.28, and S = 0.05 $mg \cdot (g\ soil^{-1}) \cdot year^{-1}$, plus a complete complement of micronutrients; Scotts-Sierra Horticultural Products Co., Marysville, Ohio) when containers were filled or transplanted to larger pots. Trees received deionized water as needed. Seedlings were transplanted into ~13-L citrus containers in March 1998 (McConkey Co., Garden Grove, Calif.) and again into ~43-L containers in June 1999. Containers were periodically moved within chambers to avoid location effects.

**Herbivory trial**

The herbivory experiment was initiated in July 1999; thus, the cuttings were ~38 months old and had been receiving their respective $CO_2$ treatments for ~18 months. Needles from current year growth, including fascicle sheaths, were removed from sample trees. Needles were cut to fit into 10 cm plastic Petri dishes (fascicle sheaths were not included), and needle fresh mass was recorded. Moistened cotton balls were wrapped at the cut ends of needles to prevent desiccation.

Sawfly larvae were obtained from a natural infestation of a nearby *Pinus palustris* Mill. tree. Approximately 1 week after hatching, larvae were weighed and randomly assigned to a treatment group, that is, one larva per Petri dish containing needles from one experimental tree kept at room temperature. Ten larvae were used to generate fresh to dry mass conversions at the beginning of the feeding trial; 72 additional larvae received new needles every other day or ad libitum as the experiment progressed. Uneaten needles were collected, dried, and weighed for calculating consumption. Frass (larval waste) was collected periodically and dried and weighed at the experiment's end.

To calculate growth and digestion indices, we randomly selected one third of the original 72 larvae after 10 days for determining dry mass. Using mass gain data and frass mass, we calculated growth and digestive indices for these same larvae. Relative growth rates (RGRs), relative consumption rates (RCRs), efficiency of conversion of ingested (ECI) and digested (ECD) food, and approximate digestibility (AD) were calculated on the basis of Waldbauer (1968):

$$\mathrm{RGR} = \frac{\text{larval mass gain}}{\text{mean larval dry mass} \times \text{time}}$$

$$\mathrm{RCR} = \frac{\text{dry mass of needles consumed}}{\text{mean larval dry mass} \times \text{time}}$$

$$\mathrm{ECI} = \frac{\text{larval dry mass gain}}{\text{dry mass of needles consumed}} \times 100$$

$$\mathrm{ECD} = \left( \frac{\text{larval dry mass gain}}{\text{dry mass of needles consumed} - \text{dry mass of frass}} \right) \times 100$$

$$\mathrm{AD} = \left( \frac{\text{dry mass of needles consumed} - \text{dry mass of frass}}{\text{dry mass of needles consumed}} \right) \times 100$$

For obtaining developmental data, the remaining 48 larvae were allowed to feed until pupation and to emerge as adults. Cocoons were weighed approximately 24 h after being spun. Adults were allowed to emerge in their Petri dishes. Once adults died, they were stored in 70% ethanol in glass vials. They were later oven-dried to constant mass.

**Foliar chemistry**

Toward the end of the herbivory trial, needles were collected for chemical analyses. Fascicle sheaths were removed, and needles were weighed and then frozen in liquid nitrogen. Needles were kept at –80 °C in a freezer until lyophilization. Needles were then ground in a Retsch grinder

(F. Kurt Retsch GmbH and Co. KG, Haan, Germany) to pass through a 0.2-mm sieve and stored in plastic containers at room temperature.

Needle C and N concentrations were determined with a Fison NA1500 CN analyzer (Fison Instruments Inc., Beverly, Mass.) and expressed as percentage of dry mass. Percentage water was calculated from the fresh and dry mass of needles (Percentage water = [(fresh mass – dry mass)/fresh mass] × 100).

A modified Folin–Denis method was used to determine total phenolics in needles (Davis et al. 2001). Dried, ground needles (two replicates of 40 mg for each study tree) were extracted in 500 μL of 70% acetone (*v*/*v*) for 30 min in a sonicator. After the samples were centrifuged for 2 min at 5000 rpm, 15 μL of the supernatant was added to 5 mL deionized water in a test tube and vortexed. An aliquot of 2 mL from this mixture was added to an additional test tube. After 2 mL of Folin–Denis reagent was added to this tube, it was vortexed and allowed to stand for 3 min. Next, 2 mL of sodium carbonate was added to the tube, which was vortexed and kept at room temperature for 2 h. Absorbance was then read at $\lambda$ = 725 nm in a Spectronic spectrophotometer (Milton Roy, Rochester, N.Y.) for each of two replicates; the values were averaged for each sample.

The *n*-butanol test was used to analyze condensed tannins (Waterman and Mole 1994). Extraction consisted of 40 mg of dried, ground plant material in 1.7 mL of 70% methanol (*v*/*v*); the mixture was sonicated for 30 min. Samples were centrifuged for 2 min at 5000 rpm, after which 35 μL of the supernatant was added to 7 mL of the *n*-butanol reagent in a test tube and vortexed. Tubes were incubated for 40 min at 95 °C. After the tubes cooled, absorbance was read at $\lambda$ = 550 nm.

The ability of tannin extracts to precipitate proteins was measured by the radial diffusion assay (Hagerman 1987). Reagents were prepared according to Waterman and Mole (1994). Agar infused with bovine serum albumin (a protein) was prepared, poured into Petri dishes, and allowed to set. Six wells (5-mm diameter) were cut in the agar of each plate with a cork borer. As with the Folin–Denis method, 40 mg of dried, ground plant material was extracted in 300 μL of 70% acetone. After centrifugation, 36 μL of the supernatant was added to each well. There were two replicates for each needle sample: that is, the extraction for each tree was tested on two plates. Plant samples from the six trees representing one $CO_2$ chamber were also randomly assigned well positions in the Petri dish. The replication and randomization helped minimize effects of well depth variation from plate to plate. Plates were covered with Parafilm (American National Can, Inc., Menasha, Wis.) and stored at 30 °C for 3 days. The diameter of the precipitation ring was measured twice at perpendicular angles, and the values were averaged. The area for each ring was calculated, and then the areas of the replicate rings were averaged. The ring area relates to the amount of tannin in the plant extract (Hagerman 1987). We did not use standards (e.g., quebracho tannin) to quantitate defense compounds in the total phenolics, condensed tannins, or protein precipitation tests.

Attempts to analyze for resin acids by gas chromatography were unsuccessful. We used a modification of Greff and Ericson (1985), but the signals in the range for resin acids were weak and inconsistent, perhaps as a result of the storage method or age of needle samples.

### Statistics

Data were analyzed using the mixed model procedure of SAS (Littell et al. 1996). The random variables were block and block × $CO_2$. Least square means were used to determine significant differences among families; these results are denoted by Pr > *t*. The difference between treatment means was considered statistically significant at $\alpha \leq 0.05$. Trends were noted at $0.05 < \alpha < 0.15$. Three-way interactions were infrequently significant and are not discussed. Raw data did not violate the basic assumptions of analysis of variance (ANOVA).

The radial diffusion assay and colorimetric assays (i.e., Folin–Denis and *n*-butanol) are useful for determining relationships between treatments but are of limited value for determining quantities of tannins. Therefore, we did not attempt to convert those data into quantitative measures of tannins. Radial diffusion data were analyzed as precipitation ring areas and total phenolics (Folin–Denis), and condensed tannins data (*n*-butanol) were analyzed as raw absorbances (Davis et al. 2001).

Days to pupation and days to emergence were analyzed with the Kaplan–Meier test for survival analysis and the Peto-Peto–Wilcoxon rank test, which shows significance between treatments (SAS Institute Inc. 1998). Simple linear regressions were used to determine relationships between foliar chemistry and insect performance. Regressions were performed by $CO_2$, that is, regressing all the elevated values and then regressing all the ambient values to obtain one regression equation for each $CO_2$ treatment. Most of the insects in this study were females, and days to pupation, pupal mass, days to emergence, and the values for adult dry mass were analyzed without regard to sex. Analyzing males and females separately did not yield significantly different results from those obtained by analyzing sexes together (ANOVAs using sex as a variable did not yield *P* values below 0.05).

## Results

### Needle chemistry

A number of needle characteristics changed as a result of the experimental treatments or family differences. Needle N concentration was significantly affected by all three factors in our experiment (Table 1). The N concentration was 27% higher in ambient-$CO_2$ treatments than in elevated-$CO_2$ treatments and 26.4% higher in high-N fertilization treatments than in low-N fertilization treatments. There was also a significant $CO_2$ × N interaction for N concentration. High-N trees exhibited a greater decline in foliar N concentration in elevated $CO_2$ than did low-N trees; thus, the interaction was based on a difference in magnitude of response. Both the susceptible (Pr > *t* = 0.003) and resistant (Pr > *t* = 0.0007) families had higher needle N concentrations than differential (having intermediate resistance to fusiform rust) needles, but the differences were not large (~7% in both cases).

**Table 1.** Foliar chemistry values of *Pinus elliottii* needles that were fed to *Neodiprion lecontei* larvae.

| $CO_2$ ($\mu L \cdot L^{-1}$) | N (mg $N \cdot g^{-1}$) | Family (resistance level) | Needle N (% of dry mass) | Needle C (% of dry mass) | C/N ratio | Water content (% fresh mass) | Total phenolics (absorbance at $\lambda$ = 725 nm) | Condensed tannins (absorbance at $\lambda$ = 550 nm) | Protein precipitation (area of rings) ($mm^2$) |
|---|---|---|---|---|---|---|---|---|---|
| 365 | 0.02 | S | 0.67 | 46.0 | 69.97 | 62.3 | 0.408 | 0.43 | 67.1 |
| | | D | 0.65 | 46.0 | 70.62 | 61.8 | 0.430 | 0.46 | 70.0 |
| | | R | 0.70 | 46.2 | 66.44 | 60.5 | 0.475 | 0.47 | 76.6 |
| 365 | 0.2 | S | 0.94 | 46.6 | 49.98 | 63.0 | 0.370 | 0.36 | 68.4 |
| | | D | 0.82 | 46.1 | 56.35 | 63.5 | 0.392 | 0.40 | 64.8 |
| | | R | 0.91 | 46.7 | 51.50 | 61.4 | 0.423 | 0.44 | 71.3 |
| 720 | 0.02 | S | 0.58 | 45.3 | 79.17 | 59.9 | 0.428 | 0.38 | 62.1 |
| | | D | 0.54 | 45.7 | 85.48 | 58.4 | 0.479 | 0.49 | 66.9 |
| | | R | 0.57 | 45.6 | 80.52 | 57.8 | 0.492 | 0.48 | 79.1 |
| 720 | 0.2 | S | 0.66 | 46.0 | 69.66 | 53.6 | 0.399 | 0.46 | 76.1 |
| | | D | 0.65 | 45.9 | 71.09 | 58.6 | 0.412 | 0.49 | 72.1 |
| | | R | 0.70 | 46.0 | 66.30 | 58.1 | 0.480 | 0.49 | 75.6 |
| ANOVA | | $CO_2$ | $P < 0.001$ | $P = 0.017$ | $P < 0.001$ | $P < 0.001$ | $P = 0.069$ | $P = 0.307$ | $P = 0.308$ |
| | | Family | $P = 0.001$ | $P = 0.285$ | $P = 0.005$ | $P = 0.603$ | $P < 0.001$ | $P = 0.011$ | $P < 0.001$ |
| | | N | $P < 0.001$ | $P < 0.001$ | $P < 0.001$ | $P = 0.679$ | $P < 0.001$ | $P = 0.457$ | $P = 0.331$ |
| | | $CO_2$ × Family | $P = 0.433$ | $P = 0.318$ | $P = 0.990$ | $P = 0.503$ | $P = 0.883$ | $P = 0.579$ | $P = 0.807$ |
| | | $CO_2$ × N | $P < 0.001$ | $P = 0.828$ | $P = 0.123$ | $P = 0.131$ | $P = 0.760$ | $P = 0.027$ | $P = 0.027$ |
| | | Family × N | $P = 0.342$ | $P = 0.126$ | $P = 0.989$ | $P = 0.230$ | $P = 0.698$ | $P = 0.707$ | $P = 0.012$ |
| | | Sample size (*n*) | 72 | 72 | 72 | 72 | 72 | 72 | 72 |

**Note:** Values represent treatment means. ANOVA, analysis of variance; D, differential; R, resistant to *Cronartium quercuum* f. sp. *fusiforme;* S, susceptible.

**Table 2.** Values for larval, pupal, and adult mass of *Neodiprion lecontei* fed *Pinus elliottii* needles from various $CO_2$, N, and genetic treatments.

| $CO_2$ ($\mu L \cdot L^{-1}$) | N (mg $N \cdot g^{-1}$) | Family (resistance level) | Larval dry mass after 10 days (mg) | Dry mass gain after 10 days of feeding (%) | RGR ($g \cdot g^{-1} \cdot day^{-1}$) | RCR ($g \cdot g^{-1} \cdot day^{-1}$) | Pupal fresh mass (mg) | Adult dry mass (mg) |
|---|---|---|---|---|---|---|---|---|
| 365 | 0.02 | S | 10.5 | 563.1 | 0.1480 | 3.48 | 41.57 | 6.14 |
| | | D | 5.6 | 423.3 | 0.1411 | 3.60 | 33.27 | 5.25 |
| | | R | 6.9 | 580.1 | 0.1504 | 2.24 | 39.89 | 5.64 |
| 365 | 0.2 | S | 11.8 | 776.8 | 0.2159 | 3.63 | 41.57 | 7.56 |
| | | D | 11.5 | 651.6 | 0.1700 | 3.04 | 54.85 | 10.70 |
| | | R | 11.2 | 220.4 | 0.1681 | 3.22 | 55.14 | 9.03 |
| 720 | 0.02 | S | 5.0 | 308.2 | 0.1341 | 5.31 | 45.08 | 7.05 |
| | | D | 4.5 | 302.8 | 0.1415 | 2.74 | 29.86 | 3.80 |
| | | R | 4.5 | 147.9 | 0.0894 | 3.05 | 30.06 | 3.26 |
| 720 | 0.2 | S | 8.3 | 516.2 | 0.1624 | 2.79 | 42.07 | 6.00 |
| | | D | 7.0 | 816.9 | 0.1757 | 10.00 | 43.67 | 7.36 |
| | | R | 5.5 | 325.9 | 0.1125 | 1.75 | 47.36 | 9.38 |
| ANOVA | | $CO_2$ | $P = 0.113$ | $P = 0.149$ | $P = 0.063$ | $P = 0.053$ | $P = 0.258$ | $P = 0.173$ |
| | | Family | $P = 0.585$ | $P = 0.088$ | $P = 0.124$ | $P = 0.010$ | $P = 0.817$ | $P = 0.990$ |
| | | N | $P = 0.087$ | $P = 0.081$ | $P = 0.033$ | $P = 0.204$ | $P = 0.004$ | $P = 0.002$ |
| | | $CO_2 \times$ Family | $P = 0.899$ | $P = 0.416$ | $P = 0.199$ | $P = 0.041$ | $P = 0.314$ | $P = 0.639$ |
| | | $CO_2 \times$ N | $P = 0.576$ | $P = 0.139$ | $P = 0.716$ | $P = 0.354$ | $P = 0.664$ | $P = 0.758$ |
| | | Family × N | $P = 0.844$ | $P = 0.129$ | $P = 0.696$ | $P = 0.007$ | $P = 0.032$ | $P = 0.053$ |
| | | Sample size ($n$) | 24 | 24 | 24 | 24 | 42 | 39 |

**Note:** Values represent treatment means. ANOVA, analysis of variance; D, differential; R, resistant to *Cronartium quercuum* f. sp. *fusiforme;* RCR, relative consumption rate; RGR, relative growth rate; S, susceptible.

Carbon dioxide and N level affected needle C concentration, though the absolute differences were slight (Table 1). Ambient-$CO_2$ needles had a slightly higher C concentration (by 1.1%) than elevated-$CO_2$ ones. The C/N ratios also showed $CO_2$, N, and family effects (Table 1). Elevated-$CO_2$ and low-N fertilization treatments both resulted in higher C/N ratios in needles. Differential needles had higher C/N ratios than both the susceptible and resistant families. Needle water content was 7% lower in elevated-$CO_2$ needles than in ambient-$CO_2$ ones (Table 1). Neither N fertilization nor family affected water content.

Levels of total phenolics were affected by all three factors (Table 1). Elevated-$CO_2$ needles tended to have higher total phenolics than ambient-$CO_2$ needles. Needles in low-N fertilization treatments also had higher total phenolics. With family, resistant needles had higher total phenolics than differential needles ($Pr > t = 0.0043$) and susceptible ones ($Pr > t < 0.0001$). Differential needles in turn had higher total phenolics than susceptible needles ($Pr > t = 0.045$). Similarly, both resistant ($Pr > t = 0.0045$) and differential needles had higher condensed tannin levels than susceptible ones ($Pr > t = 0.021$) (Table 1). Neither $CO_2$ nor N affected condensed tannin values. Protein precipitation power of tannins, which reflects the concentration of tannins in the plant extract, was also unaffected by $CO_2$ or N (Table 1). Family did affect protein precipitation power; resistant needle extracts produced larger rings than differential needle extracts ($Pr > t = 0.0002$) and susceptible ones ($Pr > t = 0.0005$) (Table 1).

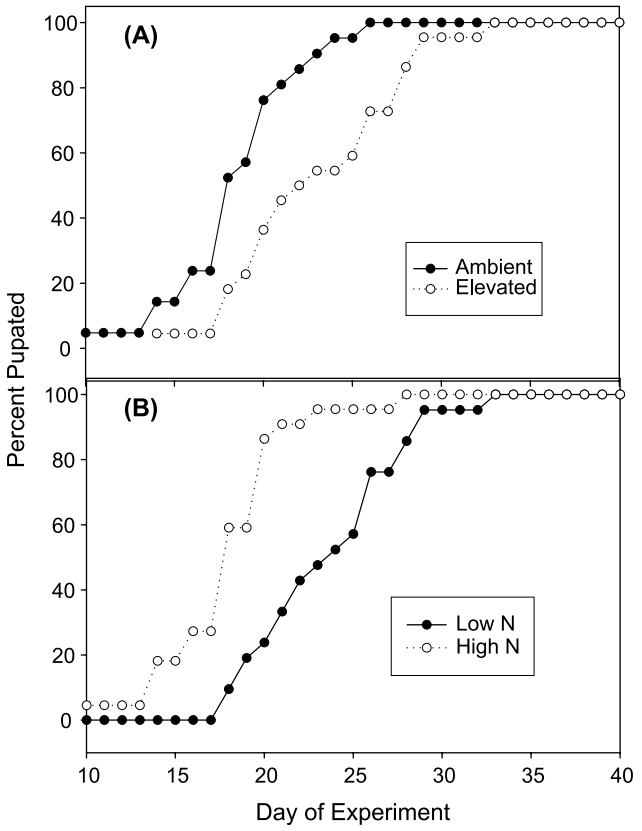
## Insect performance

The values for initial larval mass were evenly distributed among treatments; that is, there were no differences among $CO_2$, N, or family treatments when the feeding trial began. Dry mass of larvae fed on ambient-$CO_2$ needles for 10 days tended to be higher (65.1%) than that of larvae fed elevated-$CO_2$ needles (Table 2). There was also a trend toward higher values for larval dry mass in those fed high-N needles than in those fed low-N needles (49.5%). Family had no effect on final larval dry mass. Percentage dry mass gain for the portion of larvae allowed to feed for 10 days tended to be lower for low-N treatment and resistant family needles (Table 2). Elevated $CO_2$ also tended to decrease percentage dry mass gain (Table 2).

RGR was significantly higher for larvae fed high-N needles (24.8%) than for those fed low-N needles, and RGR was higher for larvae fed ambient $CO_2$ needles (21.9%) (Table 2). Family also tended to affect RGR: larvae feeding on susceptible family needles had a 26.9% higher RGR than those fed resistant needles ($Pr > t = 0.052$).

Carbon dioxide level and family influenced RCR (Table 2). Elevated-$CO_2$ diets raised consumption rates by 33.5%, compared with ambient needles. Resistant diets reduced RCR, compared with differential needles (89.1% higher RCRs than resistant; $Pr > t = 0.003$) and susceptible ones (48.4%; $Pr > t = 0.068$). Also, larvae fed differential needles tended to have higher RCRs than susceptible-fed larvae ($Pr > t = 0.109$). The raw values for RCR of larvae fed resistant needles were lower than for those with any other factor. Significant interactions included $CO_2 \times$ family ($P = 0.041$; differential needles had much higher RCR in elevated $CO_2$ compared with other families; differential RCR was also higher in elevated than in ambient $CO_2$) and family × N ($P = 0.007$; differential, high-N needles were consumed at a faster rate than differential low-N ones and than needles in any other family × N combination). There were no signifi-

**Fig. 1.** Percentage of *Neodiprion lecontei* larvae pupated when fed *Pinus elliottii* needles under (A) ambient-$CO_2$ or elevated-$CO_2$ (365 or 720 μL·L$^{-1}$) treatments and (B) low-N or high-N (0.02 or 0.2 mg N·g$^{-1}$) fertilizer applications. Values for mean days to pupation were significantly different on the basis of $CO_2$ and N treatments ($P$ = 0.0015 and $P$ < 0.0001, respectively).

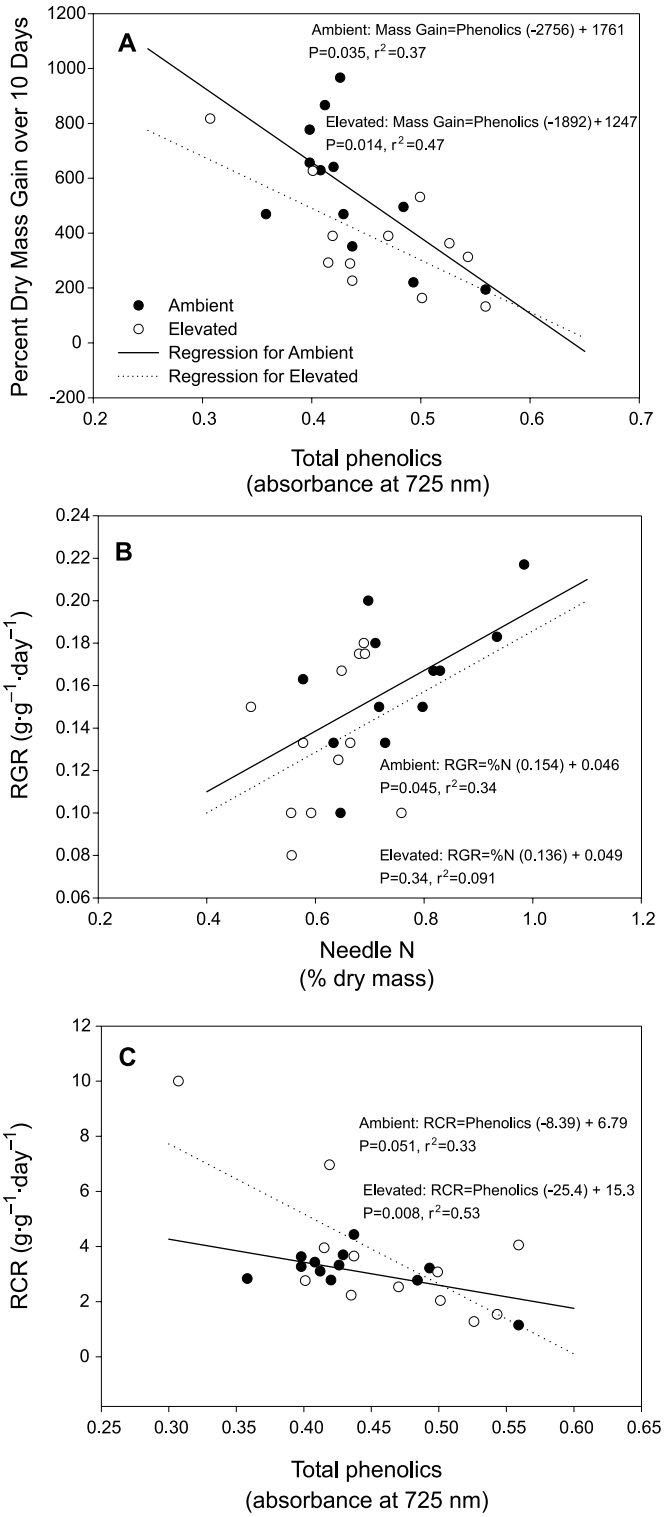



cant effects on digestion in terms of ECI, ECD, or AD by any of the main factors (data not shown).

Days to pupation increased under elevated $CO_2$ (Fig 1A). The mean days to reach pupation for larvae fed $CO_2$-enriched needles was 22.3 days (SE ± 1.0), 4.4 days longer than the mean for ambient-fed larvae of 17.9 days (SE ± 0.81). Survival analysis showed that these means were indeed significantly different ($P$ = 0.002). Larvae fed on high-N needles also took longer to reach pupation ($P$ < 0.0001) (Fig. 1B). Mean days to pupation for larvae fed on low-N needles was 23 days (SE ± 0.86), compared with 17.2 days (SE ± 0.80) for larvae fed on high-N needles, a difference of 5.8 days. Family had no effect on days to pupation ($P$ = 0.889) (data not shown). The values for pupal mass were 29.5% higher in high-N treatments, but $CO_2$ levels had no effect on pupal mass (Table 2).

Days to emergence did not follow the same trends as days to pupation; although N and family influenced emergence time, $CO_2$ did not (data not shown for emergence). Larvae fed susceptible needles tended to take longer to emerge as adults than those from resistant groups ($P$ = 0.069). Susceptible-fed larvae had a mean days to emergence of 19.6 (SE ± 0.46), compared with 18.2 days (SE ± 0.37) and 18.3 days (SE ± 0.41) for resistant and differential, respectively. Larvae fed on low-N needles took longer to emerge than lar-

**Fig. 2.** Data points and plots of regression lines for (A) *Neodiprion lecontei* percentage dry mass gain and total phenolics in *Pinus elliottii* needles; (B) RGR and needle N concentrations; and (C) RCR and total phenolics, in ambient and elevated $CO_2$. Regression equations and associated statistics are given on each graph. RCR, relative consumption rate; RGR, relative growth rate.



vae fed on high-N ones ($P = 0.017$). Low-N diets resulted in a mean days to emergence of 19.2 days (SE ± 0.29), compared with 18.3 days (SE ± 0.40) for high-N diets. Adult dry mass (both male and female) was not affected by elevated $CO_2$ or family, but low-N treatment did reduce values for adult mass (Table 2); adults in the high-N treatment weighed 61% more than adults in the low-N treatment.

Regressions yielded some significant, though not always strong, relationships between foliar chemistry and insect performance. Percentage dry mass gain, for instance, was negatively correlated with total phenolics in both $CO_2$ treatments ($P < 0.05$, $r^2$ ambient = 0.37 and $r^2$ elevated = 0.47) (Fig. 2A). RGR was positively related with N concentration ($P = 0.045$, $r^2 = 0.35$) (Fig. 2B). RCR was negatively related to the concentration of total phenolics in both ambient- and elevated-$CO_2$ tissue, but the relationship was stronger under elevated $CO_2$ where phenolics were higher (ambient, $P = 0.051$, $r^2 = 0.33$; elevated, $P = 0.008$, $r^2 = 0.53$) (Fig. 2C).

## Discussion

Insect performance declined in elevated $CO_2$, in low-N treatments, and with seedlings from the family of *P. elliottii* most resistant to fusiform rust. Some of the most dramatic changes in insect performance included reduced growth rates, increased consumption rates, and increased time to reach pupation. Larvae reared on elevated-$CO_2$ or low-N diets did not accumulate as much biomass as their counterparts in ambient-$CO_2$ or high-N treatments; despite increased consumption rates, they were also delayed in reaching the pupal phase. These negative insect responses were associated with lower N concentrations and higher phenolic levels in the needle diets. Needle N concentrations for the low-N treatment were similar to those observed with longleaf pine grown under similar conditions (Mitchell et al. 1995; Entry et al. 1998). The decline in N concentration under elevated $CO_2$ and low-N fertilization was consistent with the findings of other elevated-$CO_2$ studies (Julkunen-Tiitto et al. 1993; Tissue et al. 1997; Williams et al. 1997*b*; Lindroth and Kinney 1998). However, N concentrations for the elevated-$CO_2$, high-N treatment were lower than expected from previous experience. Most likely, the additional growth under elevated $CO_2$ caused a dilution of N and thus the lower N concentrations observed. Previous research has shown that insect digestion, growth, consumption, and population levels can be affected by plant N content (Popp et al. 1986; Wagner 1986; Lawler et al. 1997; Williams et al. 1997*a*). Here, RGR, percentage dry mass gain, and adult mass were all negatively affected by low foliar N (according to regressions).

Phenolic levels were significantly related to RGR and RCR (negative relationships by regressions) in both ambient and elevated $CO_2$. Phenolics can be a feeding deterrent for insects (Bernays and Chapman 1994) and specifically sawflies (Schafellner et al. 1999; but see Wagner 1986). However, larvae fed elevated-$CO_2$ needles (which contained higher phenolic levels) had higher RCRs than larvae fed ambient-$CO_2$ needles. This is often the case in elevated-$CO_2$ studies and may be associated with lower N content in foliage (Bezemer and Jones 1998). Despite this increased RCR, larvae fed elevated-$CO_2$ needles were not able to catch up those fed ambient-$CO_2$ needles in terms of RGR, dry mass gain, and time to pupation. Similarly, Williams et al. (1994) found that this same species of sawfly reared on elevated-$CO_2$ grown *Pinus taeda* L. needles had no increase in RGR, even when RCRs increased. This could be because, as Johnson and Lincoln (1990) suggested, compensatory feeding in high-$CO_2$ environments could expose larvae to more defense chemicals per unit N consumed.

Insects must usually attain a target body mass before initiating metamorphosis (Gullan and Cranston 2000), so it was not surprising that the decrease in larval mass we observed had implications for time to pupation. The extended larval stage in the elevated-$CO_2$ or low-N treatments of our study contrasts with the results of Williams et al. (1997*a*; again using *N. lecontei* and *P. taeda*), in which no difference in days to pupation occurred on high-$CO_2$ foliage. If larvae take longer to pupate, they are exposed for longer periods to predators, as predicted by the slow growth – high mortality hypothesis (Benrey and Denno 1997). Also, the longer it takes for adults to emerge, the more days the sawfly is in the cocoon stage and thus vulnerable to predators. The values for pupal and adult mass decreased in low-N treatments, and time to emerge as adults increased in low-N and susceptible needles. Although the issue was not examined in this study, reduced pupal and adult mass may affect sawfly reproduction (Zhang and Wagner 1991).

Larval performance on needles of the different resistance levels to fusiform rust (families) was associated with phenolic levels. Larvae fed resistant needles had lower RCRs than those fed susceptible or differential diets. This suggests that resistant needles, with their higher phenolic levels, had a deterrent effect on the larvae. Furthermore, the differential class of needles, which had intermediate levels of phenolics, caused insect responses that were intermediate to those in insects fed the resistant and susceptible needles. This supports the concept of phenolics and tannins as quantitative defense compounds; that is, their effectiveness depends on their concentration (Gullan and Cranston 2000). We observed a clear pattern with the three pine families; as the concentration of phenolics rose, larval growth rate decreased.

The family most resistant to the sawflies in our study (in terms of reduced growth rate) is also most resistant to fusiform rust. This might suggest some cross-resistance; that is, the mechanism for resisting this fungal pathogen might also confer protection against sawflies. Phenolics, which were highest in the resistant family, may play a role against fungal invaders (Legaz et al. 1998; Reddy et al. 1999). It is also possible that some other factor that rose in tandem with phenolics could be effective against both fungi and sawflies. Michelozzi et al. (1990) found a relationship between a monoterpene's concentration and resistance to fusiform rust; they suggested that the particular monoterpene may in fact be linked to a gene for something more critical in resistance. In addition to producing phenolics and condensed tannins, pines produce terpenes, such as resin acids, which may have defensive properties against herbivores (Björkman 1997). Our tests for resin acids were unsuccessful, but this is a subject that could be addressed in future studies. If the relationship in our study proves consistent — that is, that slash pine needles high in phenolics are more resistant to fusiform rust and insect pests such as sawflies — such genotypes would

be wise choices for forest managers to plant. These genotypes would also have a competitive advantage if they do not suffer as much insect damage as other genotypes.

We were also interested in the possible interactions of these three genotypes with $CO_2$ levels. Do differential genotypic responses to $CO_2$ enrichment exist? This area of $CO_2$ research is still unclear, and the answer seems to depend on the species and trait studied, with some studies reporting $CO_2$ × genotype interactions (Goverde et al. 1999; Pritchard et al. 2000; Lindroth et al. 2002); and others, none (Fajer et al. 1992; Johnsen and Major 1998). We observed a genotype × $CO_2$ interaction for RCR. Larvae showed significantly higher RCRs on differential needles in elevated $CO_2$ than those of other families in elevated or ambient $CO_2$. Perhaps RCR increased in response to the low N in differential needles (lower than in the other two families), which was further depressed in elevated $CO_2$. The phenolic levels of differential needles were lower than in resistant needles and thus possibly less deterrent to larvae. The implication for forest plantings is that not all genotypes necessarily respond equally to elevated $CO_2$. It may be useful to perform trials on a variety of genotypes to determine whether insect damage or survival would be more or less detrimental for certain genotypes in these predicted future environments.

We also observed a number of genotype (family) × N interactions. As with $CO_2$ × genotype interactions, this demonstrates that even within the same species, plants will not necessarily respond in the same way to an environmental influence, in this case, soil fertility. Insect performance could also be affected by these genotype × N interactions. In several cases (protein precipitation, dry mass gain, and pupal and adult mass), responses of the susceptible and resistant families in the high-N treatment differed in direction and (or) magnitude. In needle C, RCR, and dry mass gain, the differential family responded differently to high N than the susceptible and resistant families. These genotypic differences in varying N situations imply that, as with elevated $CO_2$, individual plants within a species can respond in ways that are more or less favorable for the survival of the plant, especially in terms of insect herbivory. This genotypic variability can be exploited in selection programs to enhance performance of slash pine plantations grown under future, altered environmental conditions.

Soil N fertility affected how plants responded to elevated $CO_2$. Several observed interactions could be related to the growth stimulus of elevated $CO_2$ and high N. Shoot/root ratios have been shown to vary greatly in response to elevated $CO_2$ (Rogers et al. 1996). Although not measured in this study, it is likely that shoot/root ratio increased, given that plants were grown in pots and may have experienced root restriction. This could explain why the decreased water content in elevated $CO_2$ was of a greater magnitude in the high-N treatment than in the low-N treatment. If root restriction caused a fertility deficit, excess carbon could be used for C-based defense compounds as well; condensed tannins and protein precipitation were significantly higher in plants exposed to elevated $CO_2$ than in those exposed to ambient $CO_2$ only when grown under high-N conditions. Elevated $CO_2$ resulted in lower needle N concentrations, regardless of soil N growth conditions. This again could be related to a fertility deficit resulting from either root restriction or increased growth under elevated $CO_2$. Thus, even when plants were supplied high soil N, elevated $CO_2$ still caused a decline in foliar N concentration. This is in agreement with the general trend reported in the literature (see review by Rogers et al. 1999). Despite the dilution of foliar N concentrations by elevated $CO_2$ in both N treatments, high-N application did increase N concentrations in both $CO_2$ treatments. Higher available soil N, as may occur with N deposition near industrial areas (Köchy and Wilson 2001) or through fertilization, could alleviate (in part) the decline in foliar N in elevated $CO_2$ and improve the diet for folivorous insects, as observed in this study and in others (Hättenschwiler and Schafellner 1999).

## Conclusions

Elevated $CO_2$ or low-N environments result in *P. elliottii* needles that are a poorer food source for *N. lecontei* larvae. Foliar N concentrations decreased and phenolic levels rose in these treatments, making these needles less nutritious and possibly more deterrent to the larvae. According to our data, *N. lecontei* larvae could be negatively impacted in these environments, leading to longer development times and lower pupal and adult mass (in the case of low N). The former could increase exposure of larvae to predators, and the latter could affect fecundity and future population size. Nitrogen inputs, such as from N fertilization or deposition, may partially counter the changes in foliar chemistry that are detrimental to insects, such as lower foliar N and higher C-based defense compounds that can occur in elevated $CO_2$. Conversely, low-N fertilization might further reduce foliar N content and raise defense compound levels in high $CO_2$, resulting in even less nutritious and palatable foliage for insects. Also, inherent differences among plant genotypes can affect insect performance and interact with other environmental factors, such as atmospheric $CO_2$ or soil N. Evolutionary success of certain genotypes may decline or be strengthened in the environment of the future. The family most resistant to fusiform rust made the poorest diet for larvae, suggesting the mechanism for resistance may be the same or related. Our study highlights the ecological impact of increasing $CO_2$ levels and N fertility; aside from having direct effects on the plant itself, they could also affect insect herbivores and possibly their predators or parasites.

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