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PLANT ANIMAL INTERACTIONS

Limited impact of elevated levels of polyphenol oxidase on tree-feeding caterpillars: assessing individual plant defenses with transgenic poplar

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Abstract Polyphenol oxidase (PPO) is commonly believed to function as an effective antiherbivore defense in plants. PPO is induced in plants following herbivory, and insect performance is often negatively correlated with PPO levels. However, induced defenses create numerous changes in plants, and very little work has been done to test the direct effects of PPO on insect herbivores separately from other changes. This study examined the impacts of high levels of PPO on the performance of two species of tree-feeding caterpillars (*Lymantria dispar* and *Orgyia leucostigma*) on poplar. Transgenic PPO-overexpressing poplar (*Populus tremula* × *Populus alba*) was used as a source of elevated-PPO leaves, thereby controlling for the multiple effects of induction. In addition, the impacts of treating poplar foliage with high levels of purified mushroom PPO were examined on the two caterpillar species. Contrary to expectation, in several cases increased PPO levels had no significant effect on insect consumption or growth rates. Although one of the mechanisms by which PPO is believed to impact herbivores is via increased oxidative stress, the ingestion of large amounts of PPO had little or no effect on semiquinone radical and oxidized protein levels in the gut contents of lymantriid caterpillars. PPO activity in caterpillars is likely limited by the low oxygen and high ascorbate levels commonly found in their gut contents. This study questions whether induced PPO functions as an effective post-ingestive defense against tree-feeding caterpillars, and indicates that controlled, mechanistic studies are needed in other plant–herbivore systems to test for a direct effect of PPO on insect performance.

Keywords Polyphenol oxidase · Plant defense · Poplar · Transgenic trees · Herbivore

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Introduction

Although polyphenol oxidase (PPO) is widely produced by plants, the ecological and physiological roles played by PPO are still not completely clear (Mayer 2006). PPO has been postulated to serve as an important component of plant defenses against insect herbivores (Felton et al. 1989; Gatehouse 2002) and is also believed to function in wound healing, stress tolerance and pathogen resistance (e.g., Li and Steffens 2002; Thipyapong et al. 2004a). When leaf tissues are damaged, PPO is released from chloroplasts, oxidizing ortho-dihydroxy and some trihydroxy phenolic compounds to ortho-quinones in the presence of oxygen (Reaction 1). The subsequent binding of quinones to

$$\text{Phenolic} + \frac{1}{2}\ \text{O}_2 \rightarrow \text{Quinone} + \text{H}_2\text{O} \qquad (1)$$

essential amino acids in the insect gut would ruin their nutritional value, thereby decreasing insect performance (Felton et al. 1989, 1992). Additional evidence that PPO

acts as an anti-herbivore defense has come from studies demonstrating: (1) that PPO activity is induced in many plants following mechanical wounding, insect herbivory, or defense signals such as methyl jasmonate; or (2) that insect performance is negatively associated with induced PPO levels (Felton et al. 1989; Duffey and Stout 1996; Karban and Baldwin 1997; Constabel and Ryan 1998; Constabel et al. 2000).

Studies testing insect performance on plants with increased or decreased PPO levels have been done primarily with species in the Solanaceae (tomato, potato and tobacco) (Felton et al. 1989; Stout et al. 1998; Ren and Lu 2006). The insect species tested have mainly been noctuid caterpillars, but caterpillars of *Manduca sexta*, *Epirrita autumnata* and *Malacosoma disstria*, and the beetle *Leptinotarsa decemlineata* have also been examined (Castanera et al. 1996; Redman et al. 2001; Wang and Constabel 2004; Ruuhola and Yang 2006). While most of these examples demonstrated negative associations between performance and PPO levels, there are also examples in which a lack of association or even a positive association between performance and PPO levels was found (Cipollini and Redman 1999; Kessler and Baldwin 2002). The interpretation of these correlative studies is greatly complicated by the fact that concentrations of many secondary chemicals and nutrients, as well as enzymes, change when plant defenses are induced by herbivory (Hermsmeier et al. 2001; Major and Constabel 2006). It is, therefore, impossible to evaluate the effects of PPO separately from the effects of other potential defenses that are induced. To our knowledge, only one controlled study has been published testing the direct effects of PPO on herbivore performance (Wang and Constabel 2004). The results of this study on *Malacosoma disstria* (forest tent caterpillar) were mixed, with negative effects of elevated-PPO poplars found only on the growth rates of larvae reared from older egg masses.

This paper reports a study of the effects of ingested PPO on the performance (relative consumption and growth rates) of two species of tree-feeding caterpillars, *Lymantria dispar* (gypsy moth) and *Orgyia leucostigma* (white-marked tussock moth), that consumed poplar foliage. Both are common generalist species in the deciduous forests of Europe or eastern North America, and both have been used in numerous ecological and physiological studies of plant–herbivore interactions. In this study, the amount of PPO ingested by the caterpillars was increased by feeding them the foliage of transgenic PPO-overexpressing poplar (*Populus tremula* × *Populus alba*) or poplar leaves treated with purified mushroom PPO, thus avoiding the confounding effects of uncontrolled changes in other defenses and nutrients that occur following herbivory. The increases in PPO activity in both types of experiments were comparable to some of the highest levels found in induced plants (Constabel and Ryan 1998).

In addition, we have assessed the effect of ingesting elevated levels of PPO on oxidative stress in the midgut contents of the two caterpillar species. Oxidative stress in insect herbivores can be caused by the production of reactive oxygen species, such as semiquinone and other reactive free radicals, during the oxidation of phenolics in the gut (Barbehenn et al. 2005) and/or the redox cycling of quinones (Duffey and Stout 1996). Semiquinone radicals and protein carbonyls were measured as markers of oxidative stress. PPO directly produces quinones, but indirectly produces semiquinone radicals via Reaction 2 (Korytowski et al. 1987). The formation of both of these phenolic oxidation products would, over time, decrease levels of ascorbate and ascorbyl radicals (produced by the oxidation of ascorbic acid) via Reactions 3–5 (Yamasaki and Grace 1998; Barbehenn et al. 2003). Thus, the measurement of free radicals with electron paramagnetic resonance (EPR) spectrometry provides a semi-quantitative measure of the oxidative status of the midgut, indicating whether PPO produces an abundance of semiquinone radicals (oxidative stress), or whether the midgut contains relatively harmless ascorbyl radicals. Protein carbonyls can be produced by several oxidative mechanisms associated with phenolic oxidation (e.g., Burcham and Kuhan 1996).

$$\text{Quinone} + \text{Phenolic}^- \rightarrow \text{SQ}^{\cdot -} + \text{SQ}^{\cdot -} + \text{H}^+ \quad (2)$$

$$\text{Quinone} + \text{AH}^- \rightarrow \text{SQ}^{\cdot -} + \text{A}^{\cdot -} + \text{H}^+ \quad (3)$$

$$\text{SQ}^{\cdot -} + \text{AH}^- \rightarrow \text{Phenolic}^- + \text{A}^{\cdot -} \quad (4)$$

$$\text{A}^{\cdot -} + \text{A}^{\cdot -} + \text{H}^+ \rightarrow \text{DHA} + \text{AH}^- \quad (5)$$

[In Reactions 2–5, Phenolic^- and AH^- represent the phenolate and ascorbate mono-anions predominantly found at physiological pH in the caterpillar midgut (ca. pH 9–10). $\text{SQ}^{\cdot -}$ and $\text{A}^{\cdot -}$ represent semiquinone and ascorbyl radicals, respectively. DHA is dehydroascorbate, the unstable end product of ascorbate oxidation.]

Materials and methods

Plants

PPO-overexpressing *P. tremula* × *P. alba* lines were produced from clone INRA717I-B4, as described previously (Constabel et al. 2000; Wang and Constabel 2004). For

simplicity, lines 10, 19, and 21 are presented as genotypes 1, 2, and 3. High PPO activity in these plants was the result of expressing the poplar leaf PtdPPO-1 gene (Constabel et al. 2000) under the control of the cauliflower mosaic virus double 35S promoter and alfalfa mosaic virus translational enhancer. Plantlets were propagated in vitro or as green cuttings, and rooted in potting mix. Three independently transformed genotypes with high PPO activities were chosen for bioassays with insects and shipped from the University of Victoria to the University of Michigan. Untransformed parental stock plants served as the control genotype. Saplings were grown in a greenhouse in 8-l Treepots (Hummert International, Earth City, Mo.) in Sunshine potting mix (type 4), with Osmocote slow-release fertilizer (14-14-14) (Scotts, Marysville, Ohio) and watering as needed. Growth lights with 400 W high-pressure sodium bulbs (PL Light Systems, Beamsville, Ontario) were used from September to May (16 h light:8 h dark). Saplings were grown to a height of at least 1 m over a period of 3–4 months before their use in experiments. Upon completion of the experiments, all saplings were autoclaved.

Leaves were designated by the leaf plastochron index (LPI), defining the first leaf with a length ≥2 cm as LPI 0 (Wang and Constabel 2004). Leaves for experiments were cut from LPI 10–20 with a sterile razor blade, using at least three trees of each genotype. Two to three fully expanded leaves were taken per day from each tree, beginning with the lowest LPI. This sampling method has no significant effect on PPO induction in this poplar hybrid (J. Wang and C. P. Constabel, unpublished data). After washing the leaves for 20 min in a water bath, their surfaces were blotted dry with paper towels. Leaf disks were cut with a cork borer (23 mm diameter), producing approximately 20 disks per leaf. All disks within each genotype were mixed to randomize potential effects of leaf position and individual tree.

Insects

L. dispar and *O. leucostigma* are in the family Lymantriidae. *L. dispar* feeds on spring foliage for a single generation, whereas *O. leucostigma* has multiple generations per year that feed on young or mature leaves. Eggs of these species were obtained from the United States Department of Agriculture (Otis Air Force Base, Mass.) and the Canadian Forest Service (Sault Sainte Marie, Ontario), respectively. All larvae were reared on an artificial diet (Barbehenn et al. 2001) in petri dishes kept in incubators at either 18 or 23°C to regulate their developmental rates. Newly molted fourth-instar *L. dispar* and final-instar *O. leucostigma* larvae were used in most experiments. These similar-sized caterpillars provided a sufficient quantity of midgut contents for chemical analysis. Experiments were carried out in the winter of 2005 and spring of 2006 on the dates given.

Chemical analysis

Replicate leaf disks cut for feeding experiments were used to measure PPO activities and the chemical compositions of the four poplar genotypes. Samples from three to five dates from January to the end of April 2006 were selected for chemical analysis. This period represents the variation across the time when most experiments were performed. PPO activities were measured in extracts prepared from frozen (–80°C) leaf disks from control and elevated-PPO genotypes on three dates during the experimental period (Constabel et al. 2000). Tannins, low molecular weight phenolics and salicylates were analyzed primarily with high-performance liquid chromatography (HPLC) and a diode array detector (Barbehenn et al. 2006). Ascorbic acid was extracted from fresh leaf samples in 5% w/v metaphosphoric acid (containing 1 mM EDTA) and analyzed with reverse-phase HPLC (Barbehenn 2003). Other nutrients were measured in samples that had been lyophilized and ground to a fine powder in a dental amalgamator (Barbehenn et al. 2004). Water was measured as the difference between the fresh and dry weights of leaf disks (70°C; 2–3 days). Leaf water content was measured on 14 dates during feeding experiments (28 January–21 March), with five replicates per genotype on each date.

Ascorbate concentrations were compared in the posterior midgut contents of *L. dispar* (fourth instar; $n = 10$) and *O. leucostigma* (final instar; $n = 9$) on the control poplar genotype (12 June). Their posterior midgut contents were dissected, extracted in 5% metaphosphoric acid (300 µl), and analyzed for ascorbate with reverse-phase HPLC.

Measurements of semiquinone and ascorbyl radicals in extracts of mid-midgut contents were made using a Bruker EMX EPR spectrometer (Bruker Instruments, Billerica, Mass.) (Barbehenn et al. 2003). Standard solutions of the stable free radical, 2,2,6,6-tetramethyl-1-piperidinyloxy (TEMPO; Aldrich) were made in 70% ethanol, and two independent standards were run per day (Barbehenn et al. 2006). The area of each first derivative spectrum was quantified by double integration using WinEPR software (Bruker Instruments), and double integral values of sample spectra were expressed in nM TEMPO radical equivalents (Barbehenn et al. 2006). Several experiments produced some spectra that were too weak to integrate accurately. Therefore, for all spectra the height of the center-field peak was measured (arbitrary *y*-axis units), and regressions of peak height vs. double integral value were used to quantify ascorbyl radicals ($R^2 = 0.78$) or semiquinone radicals ($R^2 = 0.96$). Radical concentrations in gut fluid extracts are

presented, rather than their concentrations in the sample volumes, in order to compare concentrations between extracts and model reaction mixtures.

Oxidized proteins (protein carbonyls) in caterpillar midgut contents were measured using an enzyme-linked immunosorbant assay (Winterbourn and Buss 1999). Entire midgut contents were extracted in 400 µl of pH 6.5 phosphate buffer (200 mM, nitrogen purged), and stored at –80°C under a nitrogen atmosphere for 2–4 months before analysis. Total protein concentrations in the supernatant solutions were measured with the modified Bradford assay (Stoscheck 1990). Aliquots of each sample containing 7 µg of protein were placed in each well of a 96-well microtitre plate for analysis. Protein carbonyl standard curves were constructed using mixtures of reduced and oxidized bovine serum albumin (Alamdari et al. 2005).

Effects of PPO on insect performance

Transgenic poplars

To compare the performance of third-instar *L. dispar* larvae on intact plants, newly molted larvae were weighed and placed at random on separate leaves in fine nylon mesh bags, which were tied around the petioles (10 April). Trees were maintained in the greenhouse (16-h photoperiod). Five larvae were bagged (one larva per leaf) on four trees per genotype, using LPI 12–16. Trees were rotated daily to minimize positional effects. Frass was recovered from each bag at the end of the third instar and dried at 70°C. Larvae were frozen upon molting to the fourth instar, and then dried to determine their final weights. Larval fresh weights were converted to dry weights using fresh weight:dry weight ratios from six representative larvae. Relative consumption and relative growth rates (mg/mg per day) were calculated with mean larval weights on a dry weight basis (Waldbauer 1968). To estimate consumption from the mass of feces produced by third-instar larvae, average approximate digestibilities of fourth-instar *L. dispar* on each of the four poplar genotypes were used from a separate experiment (R. V. Barbehenn, unpublished data).

Fourth-instar *L. dispar* performance was compared on leaf disks cut from each of the control and elevated-PPO poplar genotypes (28 January). Newly molted larvae were randomly assigned to each genotype (n = 10–13 larvae/genotype). Larval fresh weights were measured and converted to dry weights. Larvae were kept in individual 35-ml plastic cups in a 23°C incubator (16 h light:8 h dark). Leaves and leaf disks were cut and prepared as described above. Fresh weights of leaf disks fed to each larva were recorded, and fresh weight:dry weight ratios of representative leaf disks (n = 5) from each genotype were used to convert food weights to dry weights to measure consumption. The difference between the dry weight of the food and the dry weight of the uneaten remains was defined as the amount consumed. Fresh leaf disks were provided daily and retained their turgidity on moist filter papers. Fecal pellets were collected daily, pooled for each individual larva and dried (70°C). Upon molting to the fifth instar, larvae were frozen, dried and weighed. Consumption and growth rates were measured on a dry weight basis, as described above. An identical experiment was performed on final-instar *O. leucostigma* (14 March).

PPO-coated leaves

To examine the effect of PPO added to poplar leaves (control genotype) on larval performance, fourth-instar *L. dispar* were fed leaf disks (5 February) coated with 20 µl of a 50% acetone suspension of mushroom PPO. Control leaf disks were treated with 50% acetone. The solvent contained 5.7 mg sucrose/ml, as in other leaf-coating experiments. The PPO suspension or control solvent was spread evenly on leaf disks using an adjustable pipetter, with the pipette tip held horizontally to avoid damaging the leaf surface. PPO was applied to leaf disks at 4,467 tyrosinase U/g leaf fresh weight, according to the tyrosinase activities reported by the manufacturers. Sigma PPO (1,530 tyrosinase U/mg enzyme preparation) was used for three days, followed by Worthington PPO (836 tyrosinase U/mg enzyme preparation) on the final day. When PPO activity was later measured in the commercial PPO preparations using the same methods that we used to measure PPO in leaves (see Chemical analysis above), these activities were found to differ from the expected levels. Thus, PPO activities were increased fourfold (by 59 PPO U/g fresh weight) for 3 days, and approximately eightfold (by 103 PPO U/g fresh weight) on the final day. Larvae were weighed, assigned at random to the control (n = 10) or PPO (n = 13) treatment groups, and fed a weighed amount of poplar leaf disks. Consumption and growth rates were measured on a dry weight basis, as described above. Preliminary tests showed that 90% of the added PPO activity could be recovered from treated leaf disks 30 min after the coatings were dried (R. V. Barbehenn, unpublished data). Because poplar PPO is stable during its passage through the guts of some caterpillar species (Wang and Constabel 2004), we assumed that the added PPO protein had a negligible nutritional effect.

Phenolic-coated leaves

If PPO activity in poplar-feeding caterpillars was limited by low substrate concentration, then increasing substrate

levels would potentially increase the impact of PPO on caterpillar performance. To test this possibility, final-instar *O. leucostigma* were placed at random in one of four treatments: control genotype leaves (solvent-treated), elevated-PPO genotype leaves (solvent-treated), control genotype leaves coated with chlorogenic acid (4% dry weight), or elevated-PPO leaves coated with chlorogenic acid (4%). Chlorogenic acid was chosen as a PPO substrate because it is one of the most abundant phenolics in poplars (see "Results"), and is one of the commonly recognized substrates for PPOs (e.g., Felton et al. 1989). Chlorogenic acid (Sigma) was dissolved (30.4 mg/ml) in 50% aqueous acetone containing 5.7 mg sucrose/ml to promote feeding. A 20-µl aliquot of this solution was coated on leaf disks (cut and mixed as described above). Leaf disks from all three elevated-PPO genotypes were mixed in equal proportion. After the solvent dried on the disk surfaces (ca. 5 min), disks were fed to each larva (n = 9–13/treatment). The number of leaf disks fed to larvae was increased from three to six as consumption increased from day to day. Consumption and growth rates were measured gravimetrically (beginning March 15).

Effects of PPO on oxidative stress

Transgenic poplars

Separate experiments were performed to measure either free radicals or oxidized proteins in midgut contents. Caterpillars were placed at random on leaf disks from one of the four poplar genotypes (n = 12–16/genotype). Larvae were kept separately in 35-ml cups in a 23°C incubator. On the third day, larvae were allowed to feed on fresh disks for at least 1 h, following which they were chilled (–20°C, 6 min) and dissected under a dissecting microscope. To measure free radicals, midgut contents (20–30 mg) were placed in 300 µl of pH 10 carbonate buffer (nitrogen purged) under a nitrogen atmosphere, weighed to the nearest 0.1 mg, shaken vigorously, and centrifuged (ca. 8,000 *g*, 1 min, ambient temperature). A 200-µl aliquot of the supernatant solution was used for EPR spectrometry, as described above. All samples were scanned within 4–5 min from the time the gut contents were dissected, permitting accurate comparisons of ascorbyl or semiquinone radical concentrations in gut fluid samples (Barbehenn et al. 2003, 2005). Free radicals were measured in *L. dispar* larvae on 17 November (2005), and in *O. leucostigma* on 14 February. Oxidized proteins (protein carbonyls) were measured in the midgut contents of *O. leucostigma* on 26 April and in *L. dispar* 7 December (2005) (n = 12–16/treatment), as described above.

PPO-coated leaves

Purified mushroom PPO was applied to leaf disks at 4,467 tyrosinase U/g leaf fresh weight, as described above. For *L. dispar*, leaf disks were cut from three control trees, and mixed to randomize potential leaf position or tree effects (21 April). PPO (Worthington, 836 tyrosinase U/mg enzyme preparation) was used to increase foliar PPO activity by 103 PPO U/g fresh weight, or approximately a sevenfold increase. This experiment was repeated (18 May) with Sigma PPO (3,900 tyrosinase U/mg enzyme preparation), resulting in an increase of 139 PPO U/g fresh weight. A similar experiment was performed on *O. leucostigma* (11 July) by coating leaf disks with PPO (Sigma, 3,320 tyrosinase U/mg enzyme preparation). Ingested PPO activities were increased by 140 PPO U/g fresh weight (approximately tenfold). Free radical levels were measured in caterpillar midgut contents with EPR spectrometry as described above (n = 8–12/treatment).

Phenolic-coated leaves

L. dispar larvae were randomly assigned to feed on leaf disks from each genotype that were coated with chlorogenic acid (4% dry weight) (2 December). This experiment was conducted as described above for examining the performance of *O. leucostigma*, with the exception that each of the four genotypes was tested. Semiquinone radical levels were measured in caterpillar midgut contents with EPR spectrometry. No concurrent measurements of radical levels in larvae that ingested untreated leaf disks were made. Based on radical levels observed in ten other EPR experiments on *L. dispar* on the same hybrid poplar (R. V. Barbehenn, unpublished data), it was assumed that either very low levels of semiquinone radicals or ascorbyl radicals would be present. For comparison with the effects of chlorogenic acid treatment, the results of the nearest comparable experiment are presented (17 November; n = 10–17/genotype).

Effect of ascorbate concentration on PPO activity

The potential of ascorbate to limit PPO activity was compared in conditions found in caterpillar midgut fluid extracts (approximately a 7% v/v dilution) and in conditions that more closely approximate in vivo (undiluted) ascorbate levels. Reaction mixtures for EPR were prepared with 290 µl of pH 10 carbonate buffer (low oxygen), to which 10 µl of low-oxygen pH 4 double-distilled water (for low ascorbate treatment) or 10 µl of 4.5 mM ascorbate in pH 4 double-distilled water was added. It was calculated

that the ascorbate levels in reaction mixtures ranged from 62 to 124 μM (with no ascorbate added) and from 212 to 274 μM (150 μM ascorbate added), based on ascorbate measurements from the midguts of *L. dispar* (see below). The mid-midgut contents (ca. 25 mg) of fourth- and fifth-instar *L. dispar* larvae recently feeding on poplar (control genotype) were extracted in either the ascorbate-free or ascorbate-containing pH 10 buffer (nitrogen-purged), maintained under a nitrogen atmosphere. A 200-μl aliquot of the extract was spiked with 10 μl of a chlorogenic acid solution (14 mM in 70% low-oxygen ethanol) or double-distilled, low-oxygen water (–CGA treatment), and with 10 μl of a PPO solution (0.2 mg PPO/ml low-oxygen double-distilled water) or double-distilled, low-oxygen water (–PPO treatment). PPO (Sigma) had an activity of 1,530 tyrosinase U/mg protein. Reaction mixtures were mixed rapidly with a 200-μl pipetter, and transferred to a flat cell in the EPR cavity. EPR spectra were recorded beginning approximately 30 s from the start of the reaction for 3 min (two scans). All parameters were identical to those described above for other EPR experiments. Four combinations of chlorogenic acid and PPO treatments were tested, with a total of 3–7 replicates/treatment examined over a 2-day period. These experiments were designed to qualitatively test the hypothesis that ascorbate levels in the midgut contents of caterpillars can inhibit PPO activity.

Statistical analysis

Leaf chemical composition was compared across tree genotypes and sampling dates with two-way ANOVA (PROC MIXED) (SAS 2003). One sample representing each genotype-by-date combination was analyzed, and results were pooled across dates to simplify their presentation. Separate experiments were performed to measure the performance for each caterpillar species, and to measure levels of free radicals and oxidized proteins in their midgut contents. Individual larvae were used as replicates in all experiments. Relative growth and consumption rates were compared across tree genotypes with analysis of covariance (ANCOVA) (PROC MIXED). Models for analyzing performance used tree genotype as the main effect. In comparisons of *L. dispar* performance on intact leaves, the effects of leaf position and leaf–genotype interactions were also tested. ANCOVA models used to compare insect performance across poplar genotypes were as follows: relative growth rates (mg/mg per day) were compared using growth rate as the dependent variable and initial dry weight as the covariate. Relative consumption rates (mg/mg per day) were compared using consumption rate as the dependent variable and initial dry weight as the covariate. In all cases, models testing for a significant interaction between the dependent variable and covariate were first tested to confirm that the regression slopes were parallel. Pairwise differences between larvae on each tree genotype were examined by differences of least squares means ($P = 0.05$ for a priori comparisons or adjustment with the Tukey–Kramer method for unplanned comparisons) (SAS 2003). To test the hypothesis that larvae on the control genotype differed from larvae on the three PPO genotypes, these two groups were compared with contrasts (weighted as –3, 1, 1, 1) (PROC MIXED). Levels of free radicals and oxidized proteins were compared across tree genotypes for *L. dispar* and *O. leucostigma* with one-way ANOVA and contrasts, as described above. The normality of residuals was tested with PROC UNIVARIATE (SAS 2003). Where necessary, log or square root transformations were used to normalize residuals. Data which could not be transformed to meet the assumptions of ANOVA (radical levels in the midgut contents *L. dispar* on PPO- and chlorogenic acid-treated leaf disks) were analyzed with Kruskal–Wallis tests (Wilkinson 2000).

Results

PPO activities were substantially greater in all the elevated-PPO poplar genotypes, although the magnitude of the increase varied by experimental date. PPO activities in genotypes PPO 1, PPO 2, and PPO 3 were elevated 28-, 29- and 27-fold, respectively, on 28 January, five-, five- and ninefold on 16 March, and 12-, 14- and 26-fold on 25 April. Control genotype PPO levels were 10, 6 and 18 U/g fresh weight, respectively, on each of the examined dates. Even at their lowest levels of increase (i.e., increases of 70 U/g fresh weight), elevated-PPO poplar foliage contained higher PPO activities than would be encountered by herbivores feeding on a wide variety of other plant species (Constabel and Ryan 1998). By contrast, the elevated-PPO genotypes showed no significant differences in a variety of chemical traits that could affect the performance and gut biochemistry of caterpillars: phenolics, salicylates, ascorbate, protein, nonstructural carbohydrates and water (Table 1). Three main groups of phenolics were identified and quantified: chlorogenic acid and its derivatives (three compounds totaling 0.7% dry weight), flavonoid glycosides (nine compounds totaling 0.4% dry weight) and coumaroylquinic acid and its derivatives (three compounds totaling 0.8% dry weight). No condensed or hydrolyzable tannins were detected. Salicylates (or "phenolic glycosides") show little autoxidation at pH 10 compared with phenolic compounds (R. V. Barbehenn, unpublished data), are not substrates for PPO, and are thus reported separately from phenolic compounds. Nine salicylates were detected, with salicortin and tremulacin comprising approximately

Table 1 Chemical composition of a control genotype and elevated-polyphenol oxidase (*PPO*) poplar genotypes[a]. *DW* Dry weight, *FW* fresh weight

Genotype	Phenolics[b] (% DW)	Salicylates[c] (% DW)	Ascorbate (μmol/g FW)	Protein (% DW)	Carbohydrates[d] (% DW)	Water (% FW)
Control	1.2 ± 0.2	2.6 ± 0.3	8.2 ± 0.6	20.6 ± 0.7	7.9 ± 0.8	72.7 ± 0.3
PPO 1	1.3 ± 0.2	2.2 ± 0.1	8.6 ± 0.6	19.0 ± 0.4	6.8 ± 0.6	72.2 ± 0.5
PPO 2	1.2 ± 0.2	2.2 ± 0.2	8.3 ± 0.5	19.6 ± 0.6	7.5 ± 0.9	71.9 ± 0.4
PPO 3	1.1 ± 0.2	2.2 ± 0.1	7.9 ± 0.7	19.1 ± 0.5	6.8 ± 1.4	72.9 ± 0.5
Significance of effects						
Genotype	$P = 0.959$	$P = 0.417$	$P = 0.812$	$P = 0.371$	$P = 0.369$	$P = 0.058$
Date	$P = 0.437$	$P = 0.796$	$P = 0.014$	$P = 0.231$	$P = 0.002$	$P < 0.001$

[a] One sample per genotype was analyzed from three dates between January and April 2006 for phenolics and salicylates, four dates for ascorbate, five dates for protein, four dates for carbohydrates and 14 dates for water

[b] All measurable low molecular weight phenolics were summed: chlorogenic acid and its derivatives, coumarylquininic acid and its derivatives, and flavonoid glycosides. No tannins were detected

[c] Salicylates included primarily tremulacin and salicortin, but also seven unidentified salicylates present at 0.02–0.31% DW each

[d] Carbohydrates were present in a ratio of approximately 4:1:8:1 for glucose, fructose, sucrose and starch, respectively

70% of these compounds. Several nutrients varied significantly through time in each of the genotypes, presumably as a result of tree age or environmental changes. Ascorbate ranged from a low level of approximately 7 μmol/g fresh weight in January and February, to a high of 10 μmol/g in late April. Similarly, total nonstructural carbohydrates varied from an overall average of approximately 6% dry weight in January and February, to a high of approximately 9% dry weight in March and late April. The control genotype and elevated-PPO poplars displayed no obvious differences in growth rates or morphology, as shown previously (Wang and Constabel 2004).

To test the effects of PPO on caterpillars feeding on saplings, third-instar *L. dispar* larvae were enclosed separately in mesh bags on intact leaves. After 4–5 days of feeding, neither consumption nor growth rates differed significantly between larvae on the control genotype and the three elevated-PPO genotypes (contrast $P = 0.912$ and $P = 0.791$, respectively) (Fig. 1a, b). Both consumption and growth rates were higher on the youngest leaf than on the next older four leaves ($P < 0.050$ and $P < 0.050$, respectively), but no significant leaf–genotype interactions were found for consumption ($P = 0.855$) or growth rates ($P = 0.957$). Because PPO activities are not significantly induced by mechanical damage in the poplar hybrid used in this work (C. P. Constabel, unpublished data), PPO levels in the control and elevated-PPO genotypes were assumed to remain unchanged during this experiment.

In fourth-instar *L. dispar* that fed on leaf disks, relative growth rates were decreased by 10–16% on elevated-PPO poplar leaves compared with control genotype leaves (contrast $P < 0.001$) (Fig. 1b). Consumption rates were not significantly different between larvae on the four poplar genotypes ($P = 0.249$ across genotypes; contrast $P = 0.064$) (Fig. 1a). When high levels of PPO were coated on control genotype leaves, no effects on the consumption or growth rates of fourth instar *L. dispar* were observed ($P = 0.856$ and $P = 0.810$, respectively) (Fig. 2).

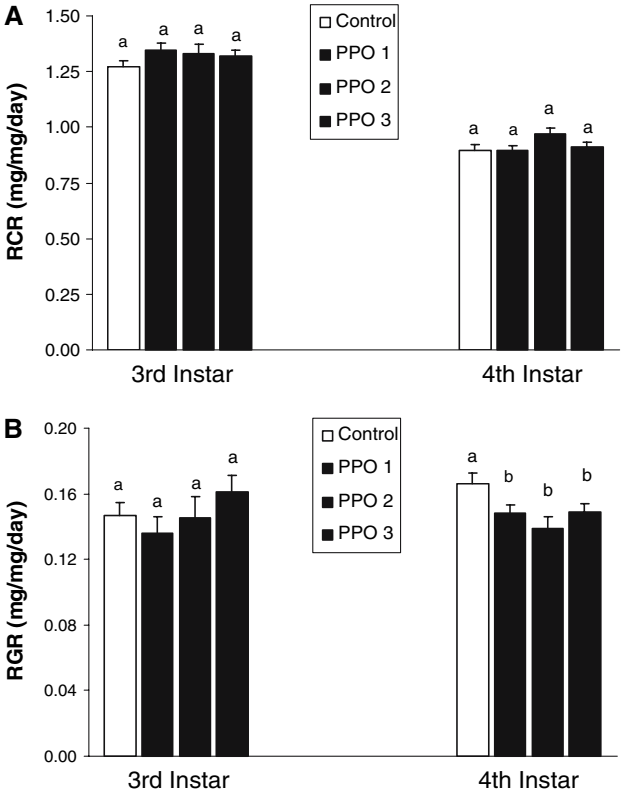

Fig. 1 **a** Relative consumption rates (*RCR*) and **b** relative growth rates (*RGR*) of third-instar *Lymantria dispar* on control and elevated-polyphenol oxidase (*PPO*) poplar saplings, and fourth-instar *L. dispar* on control and elevated-PPO poplar leaf disks. Different letters above bars designate $P < 0.05$

The performance of *O. leucostigma* larvae was unaffected by feeding on the elevated-PPO poplar genotypes

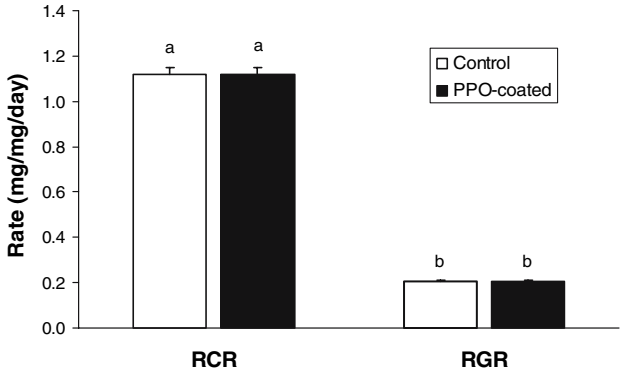

Fig. 2 RCR and RGR of fourth-instar *L. dispar* on poplar leaf disks (control genotype) coated with solvent or PPO. For abbreviations, see Fig. 1

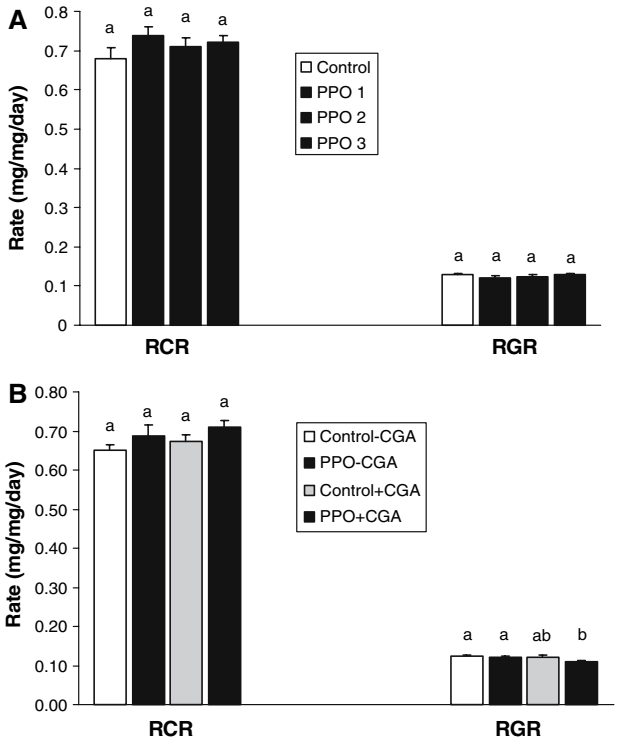

Fig. 3 **a** RCR and RGR of final-instar *Orgyia leucostigma* on control genotype and elevated-PPO poplar leaf disks. **b** RCR and RGR of final-instar *O. leucostigma* on control genotype and elevated-PPO genotype poplar leaf disks (pooled PPO genotypes) with (+) or without (–) added chlorogenic acid (*CGA*; 4% dry weight). For other abbreviations, see Fig. 1

(Fig. 3a). Consumption rates did not differ significantly across genotypes ($P = 0.747$) or in the contrast between control and elevated-PPO genotypes ($P = 0.369$). Growth rates were also similar across genotypes ($P = 0.369$) and when control and elevated-PPO genotypes were contrasted ($P = 0.400$).

If PPO from elevated-PPO poplars had little effect on caterpillars because PPO is substrate limited in the gut, then coating leaves with chlorogenic acid (4% dry weight) should produce significant PPO effects on caterpillar performance. When the performance of *O. leucostigma* was compared on the control genotype and elevated-PPO leaves, each with and without added chlorogenic acid, no significant genotype–chlorogenic acid interaction was found for consumption or growth rates ($P = 0.997$ and $P = 0.454$, respectively) (Fig. 3b). The lack of significance of the interaction effect is of primary interest because it indicates that added chlorogenic acid had no more effect on elevated-PPO leaves than it did on control genotype leaves. Growth rates of *O. leucostigma* on elevated-PPO leaves showed a nearly significant 6.5% decrease compared with larvae on the control genotype leaves ($P = 0.052$), while consumption rates did not differ ($P = 0.504$). Chlorogenic acid itself had no apparent effect on consumption or growth rates ($P = 0.497$ and 0.290, respectively).

The potential for PPO to produce oxidative stress within the insect gut was examined by measuring free radicals and oxidized proteins. *L. dispar* that fed on control and elevated-PPO poplars contained similar, low levels of semiquinone radicals in their midgut contents (58 ± 11 nM in control genotype; 62 ± 7 nM across PPO genotypes; $P = 0.594$). There was also no significant effect of elevated-PPO poplar foliage on protein carbonyl levels in *L. dispar*, which averaged 1.0 ± 0.04 and 1.0 ± 0.03 nmol/mg protein in larvae on the control genotype and across the PPO genotypes, respectively ($P = 0.198$).

In the midgut contents of *O. leucostigma*, relatively high ascorbyl radical concentrations were detected in larvae on all genotypes. However, larvae that consumed control genotype PPO poplar leaves had a significantly higher level of ascorbyl radicals (81 ± 6 nM) than did larvae on the elevated-PPO genotypes (56 ± 4 nM across genotypes) (contrast $P = 0.002$). As would be expected from the presence of high ascorbyl radical (and ascorbate) levels in the midgut contents, there was no significant difference between protein carbonyl levels in *O. leucostigma* on the control genotype (1.4 ± 0.1 nmol/mg protein) or elevated-PPO genotypes (1.5 ± 0.1 nmol/mg protein) ($P = 0.888$).

When poplar leaves were treated to increase PPO levels sevenfold (using Worthington PPO in April), ascorbyl radicals in *L. dispar* midgut extracts decreased 40% from 177 ± 11 to 107 ± 15 nM ($P = 0.003$). However, this result was not repeatable in an experiment 1 month later using Sigma PPO with a higher specific activity (22 ± 5 vs. 14 ± 3 nM; $P = 0.324$). In *O. leucostigma*, even a tenfold increase in PPO (Sigma) produced no significant effects on ascorbyl radical levels, averaging 161 ± 16 and 151 ± 3 nM in extracts from larvae on control and elevated-PPO genotypes, respectively ($P = 0.465$). In no case

was there a shift towards the production of semiquinone radicals (oxidative stress) from PPO activity.

If ingested PPO was limited by low levels of phenolic substrates in poplar foliage (Table 1), then the ingestion of high levels of chlorogenic acid should have produced a significant PPO effect on semiquinone radical levels (via Reactions 1 and 2). However, the midgut contents of *L. dispar* larvae that fed on elevated-PPO leaves coated with chlorogenic acid contained similar levels of semiquinone radicals compared with larvae that fed on control genotype leaves that were coated with chlorogenic acid (Fig. 4a) (contrast $P = 0.681$). In the absence of added chlorogenic acid, at most, only low levels of semiquinone radicals were produced in *L. dispar* on control and elevated-PPO genotypes.

Midgut fluid extracts from *L. dispar* that were spiked with chlorogenic acid (0.64 mM final concentration) produced oxidative stress, i.e., switched from ascorbyl radicals to semiquinone radicals (Fig. 4b). Contrary to expectation, the addition of PPO to extracts also caused a switch from ascorbyl to semiquinone radicals, the amounts of which were greatly increased by adding both PPO and chlorogenic acid together. However, when ascorbate was present at an average concentration of approximately 240 μM it inhibited both the oxidation of added chlorogenic acid and the ability of PPO to generate high steady-state levels of semiquinone radicals. Ascorbate concentrations in the posterior midgut contents of *L. dispar* (585 ± 51 μM) and *O. leucostigma* (934 ± 81 μM) were substantially greater than 240 μM, and levels in *O. leucostigma* were significantly greater than those in *L. dispar* ($P = 0.002$).

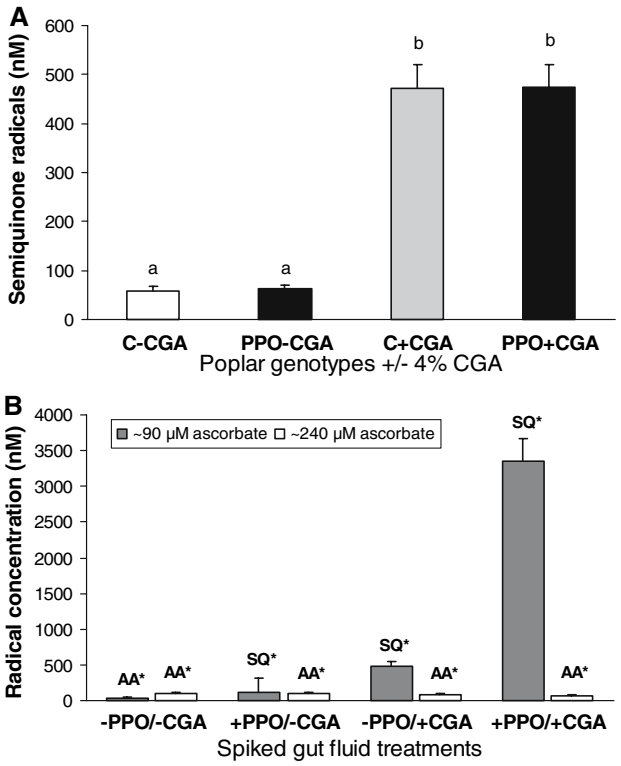

Fig. 4 **a** Composite view of semiquinone radical concentrations in the midgut contents of fourth-instar *L. dispar* after feeding on control or elevated-PPO genotypes without added CGA (4% dry weight) (November 17) or on control or elevated-PPO genotypes treated with CGA (4% dry weight) (December 2). **b** Effect of ascorbate concentration on PPO activity in reaction mixtures containing *L. dispar* midgut fluid from poplar-feeding larvae. pH 10 buffered extracts were spiked with PPO and/or CGA. *AA** Ascorbyl radicals, *SQ** semiquinone radicals; for other abbreviations, see Figs. 1 and 3

Discussion

Numerous correlative studies have been done on the potential importance of PPO as an antiherbivore defense in both ecological and agricultural systems. Yet few studies have examined the efficacy of the induced levels of this putative defense that commonly follow herbivory. While PPO is undoubtedly an important component of the suite of defense-related proteins that are induced in poplar and many other plants by insect herbivores, the results of this study suggest that the efficacy of PPO as a defense against caterpillars may be much weaker than has been believed. Overall, increased levels of either poplar PPO or mushroom PPO produced only limited effects on the performance and oxidative stress in two tree-feeding caterpillar species. In one striking example, *L. dispar* larvae fed continuously on the intact leaves of elevated-PPO saplings with no effect on their consumption or growth rates. If PPO remained active in caterpillars, it would be expected to produce a substantial increase in semiquinone radicals in their midgut contents, as was observed in pH 10 buffer (e.g., Fig. 4b; low ascorbate series). Instead, we typically observed ascorbyl radicals (produced by the oxidation of ascorbic acid) in caterpillar midguts. In no case did the ingestion of increased levels of PPO overwhelm the ascorbate antioxidant defense system, producing increased levels of semiquinone radicals (oxidative stress). These results further support the importance of the ascorbate antioxidant defense system in the gut contents of caterpillars (Felton and Duffey 1992; Barbehenn et al. 2001, 2003, 2005). The lack of effect of "induced" levels of PPO on the formation of protein carbonyls also suggests that there was not a physiologically significant increase in phenolic oxidation by ingested PPO in the gut contents of *L. dispar* and *O. leucostigma* caterpillars.

Nevertheless, the results of this study were mixed, and some results could indicate occasional negative effects of PPO on caterpillars. In one experiment, the growth rates fourth-instar *L. dispar* larvae were 11–16% lower on the elevated-PPO genotypes compared with the control

genotype, and in one experiment the growth rates of *O. leucostigma* were 6.5% lower on elevated-PPO poplar genotypes than on the control genotype. The lower levels of ascorbyl radicals in the midgut contents of *O. leucostigma* that fed on elevated-PPO poplar leaves could also indicate increased PPO activity, either in the leaf tissues during ingestion or in the gut contents following ingestion. However, where there was a decrease in performance in larvae on the elevated-PPO genotypes, the lack of change in biochemical markers of PPO activity makes it unclear what mechanism(s) could account for an effect of PPO on performance. For example, lower growth rates of fourth-instar *L. dispar* on elevated-PPO genotypes were not consistent with the apparent lack of PPO activity in their gut contents. If PPO is an effective post-ingestive plant defense, one would expect consistent congruence between PPO levels, oxidative stress and insect performance. In addition, one might also expect a greater impact on performance by high levels of PPO. In previous studies using different insect species, decreases in growth rates attributed partially or entirely to PPO have commonly been on the order of 20–50% (Felton et al. 1989; Castanera et al. 1996; Cipollini and Redman 1999; Wang and Constabel 2004; Ren and Lu 2006).

At least four factors potentially limit PPO activity in the gut contents of caterpillars: low levels of oxygen, high levels of ascorbate, high pH, and limited leaf tissue maceration. Ambient oxygen levels of about 150 mm Hg fall quickly to between 0 and 6 mm Hg in the foreguts and midguts of most caterpillars (Johnson and Barbehenn 2000). It remains to be shown whether higher oxygen levels in the unusually large foreguts of *L. dispar* (e.g., 32 mm Hg in larvae fed artificial diet) permit higher levels of oxidation in this region. Ascorbate in in vitro reaction mixtures at approximately 200–500 μM, can chemically reduce quinones and semiquinone radicals, thereby limiting the effectiveness of PPO as an oxidative defense (Janovitz-Klapp et al. 1990; Felton and Duffey 1992). Ascorbate levels measured in the midgut contents of both caterpillar species in this study were well above these lower limits. The high pH found in caterpillar midguts could also decrease the activities of ingested PPO by over 50% (Felton et al. 1989; Wang and Constabel 2003). Finally, many caterpillars feed by rapidly snipping and swallowing leaf pieces without macerating them (Barbehenn 1992). Thus, ingested leaf tissues not only pass into a low-oxygen environment in a matter of seconds, but much of the ingested leaf tissues remain structurally intact, separating PPO and phenolic substrates until cell membranes rupture inside the gut.

In addition, the induction of high levels of PPO might not produce increased PPO activity in caterpillars if foliage contained inadequate amounts of phenolic substrates. The addition of chlorogenic acid to poplar leaves provided a large amount of substrate for PPO in the gut contents of *L. dispar* larvae. However, the high levels of PPO in transgenic trees did not increase the oxidation of the added phenolics, and instead produced similar semiquinone radical levels in larvae on the control and elevated-PPO genotypes. This result suggests that induced levels of PPO in poplar leaves would not increase the level of defense against poplar-feeding caterpillars, at least not as a result of post-ingestive effects on oxidative stress. It remains possible that other markers of PPO activity, such as levels of quinones or quinone-bound proteins, would provide more conclusive evidence for PPO activity in the guts of caterpillars than measures of oxidative stress, but in either case a substantial impact on herbivore fitness would be expected if PPO is an effective plant defense. In the case of *O. leucostigma*, there was no indication of a detrimental synergistic effect on performance from increasing both PPO and substrate levels (Fig. 3b). This finding, again, strongly suggests that ingested PPO was not limited by a lack of phenolic substrates in poplar leaves. Both the relatively low levels of semiquinone radicals formed and the similarity of these levels across low and high PPO genotypes (Fig. 4a) suggest that the chlorogenic acid-treated leaves produced increased non-enzymatic phenolic oxidation (e.g., with Fe^{3+} or O_2). Phenolic oxidation is promoted by the high pH of caterpillar midguts (ca. pH 9–10) when either low levels of ascorbate, high levels of phenolics, or highly reactive types of phenolics are present (Barbehenn et al. 2003, 2005, 2006).

If PPO-catalyzed reactions in the gut contents of caterpillars are limited by low oxygen levels and high ascorbate levels, previous work on mechanisms of activity of PPO in caterpillars would need to be re-assessed. In the seminal studies on PPO as an antiherbivore defense, ambient oxygen levels and ascorbate-free buffers were used to model the effects of PPO in caterpillar gut contents (e.g., Felton et al. 1989, 1992). In addition, previous estimations of the extent of PPO-catalyzed reactions in the gut were based on levels of protein-bound quinones in the feces (Felton et al. 1989). Since PPO survives transit through the guts of some caterpillars and remains active in their feces (Wang and Constabel 2004), it is likely that measurements of quinone-bound proteins in fecal samples were increased both by enzymatic and non-enzymatic oxidation at ambient oxygen levels. The need to confirm previous work on oxidation products in fecal pellets is also supported by our observations that the bright green contents of the midgut rapidly turn brown when exposed to air (i.e., oxygen) either in the rectum or after fecal pellets are produced (R. V. Barbehenn, personal observation).

Indirect support for PPO as an antiherbivore defense has come from the observation that PPO is co-induced with a suite of anti-nutritive proteins, all of which are upregulated

by a herbivore-stimulated signaling pathway (e.g., Bergey et al. 1996; Ren and Lu 2006). In many poplars, leaf damage induces PPO along with other suspected and confirmed defense proteins, such as protease inhibitors (Major and Constabel 2006). However, in many species there is some overlap in signaling of insect and pathogen defense responses (e.g., Mewis et al. 2006). Thus, it is possible that PPO contributes to defense against plant pathogens. The importance of PPO as an antimicrobial defense is supported by the observation that resistance to pathogenic bacteria is directly affected by PPO levels in tomato (Li and Steffens 2002; Thipyapong et al. 2004b), and that PPO is induced by fungal pathogens (e.g., Raj et al. 2006). Therefore, it is possible that one role of PPO is to inhibit opportunistic pathogens that enter leaves at wounds created by herbivores. We are currently investigating pathogen resistance in elevated-PPO poplar.

While the conclusions of our work on lymantriid caterpillars do not necessarily apply to all other insect herbivores, this study does suggest that controlled, mechanistic studies are needed to test for a direct effect of PPO on insect performance in these other systems. Plant–herbivore interactions in which PPO may be more likely to function as an antiherbivore defense include those in which the herbivore species are more susceptible to plant oxidative defenses than are lymantriid caterpillars (e.g., *M. disstria*) (Wang and Constabel 2004; Barbehenn et al. 2005). It is also possible that first-instar larvae are more susceptible to the potential effects of PPO, although the seminal work of Felton and colleagues (1989) suggested that both neonate and mature larvae would be susceptible. There may also be variation in the effectiveness of PPO across plant taxa depending on its site of storage. For example, the rapid release of large amounts of PPO from the glandular trichomes of many species in the Solanaceae (Yu et al. 1992) could produce greater PPO activity during ingestion than does the slower release of smaller amounts of PPO from mesophyll and other leaf tissues.

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