

Effects of Phenolic Glycosides and Protein on Gypsy Moth (Lepidoptera: Lymantriidae) and Forest Tent Caterpillar (Lepidoptera: Lasiocampidae) Performance and Detoxication Activities

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ABSTRACT Levels of phenolic glycosides and protein influence the quality of aspen leaves to herbivorous insects, and vary in relation to genetic and environmental factors. This research was conducted to assess the independent and interactive effects of phenolic glycosides and protein on the performance and detoxication enzyme activities of gypsy moths, *Lymantria dispar* (L.), and forest tent caterpillars, *Malacosoma disstria* Hübner. We fed fourth-stadium larvae aspen leaves supplemented with 0, 2, or 4% (wet weight) phenolic glycosides and 0 or 5% (wet weight) casein. We measured stadium duration, growth and consumption rates, and food conversion efficiencies. In addition, we measured the activities of three midgut enzymes likely involved in the metabolism of phenolic glycosides: β -glucosidase, esterase, and glutathione transferase. Phenolic glycosides reduced performance of both insect species in terms of increased developmental time and decreased growth rates. Casein supplementation increased growth rates of gypsy moth larvae but slightly reduced growth rates of forest tent caterpillars. Phenolic glycosides and protein did not interactively influence stadium duration or growth rates. β -glucosidase activities declined for both insect species when reared on diets with phenolic glycosides. Esterase activities were induced by phenolic glycosides only in gypsy moths, whereas glutathione transferase activities were induced by phenolic glycosides in both species. Casein supplementation had little influence on enzyme activities, and phenolic glycosides and protein interactively affected only forest tent caterpillar esterase activity.

KEY WORDS *Lymantria dispar*, *Malacosoma disstria*, detoxication, phenolic glycosides, *Populus tremuloides*, protein

IN FORESTS OF the Great Lakes Region, U.S.A., gypsy moths, *Lymantria dispar* (L.), and forest tent caterpillars, *Malacosoma disstria* Hübner, are major defoliators of quaking aspen, *Populus tremuloides* Michaux. Although aspen is considered a preferred host for both gypsy moths (Lechowicz and Mauffette 1986) and forest tent caterpillars (Batzer and Waters 1956), performance on aspen varies greatly when insects are reared on different trees (Hemming and Lindroth 1995), different clones (Hwang and Lindroth 1997), and on trees grown under various environmental conditions (Lindroth et al. 1993a, Kinney et al. 1997, Roth et al. 1997, Hemming and Lindroth 1999). During outbreaks of these insects, the amount of defoliation also varies widely among aspen trees (R.L.L., unpublished data). Intraspecific variation in insect performance on aspen has been linked to variation in foliar chemistry (Hemming and Lindroth 1995; Hwang and Lindroth 1997, 1998). Variation in chemistry has in turn been linked to factors such as genetic differences (Hwang and Lindroth 1997), resource availability (Lindroth et al. 1993a, Kinney et al. 1997, Roth et al. 1997, Hemming and Lindroth 1999), seasonal changes

(Lindroth and Hwang 1996), and defoliation (Clausen et al. 1989).

Performance of both insects species is strongly influenced by variation in levels of phenolic glycosides (Lindroth and Hemming 1990, Lindroth and Bloomer 1991, Lindroth and Weisbrod 1991, Hemming and Lindroth 1995, Hwang and Lindroth 1997). In correlative studies, differences in phenolic glycoside levels have accounted for a greater proportion of variation in performance of gypsy moths than for forest tent caterpillars (Hemming and Lindroth 1995, Hwang and Lindroth 1997). Additionally, foliar nitrogen appears to explain more variation in forest tent caterpillar performance than in gypsy moth performance (Hemming and Lindroth 1995, Hemming and Lindroth 1999).

Growth of aspen under high light, elevated CO₂, or low nutrient availability results in foliage with increased levels of phenolic glycosides and decreased concentrations of nitrogen (Kinney et al. 1997, Hemming and Lindroth 1999). Thus, we were interested in exploring the independent and interactive effects of phenolic glycosides and protein on gypsy moth and forest tent caterpillar performance. Phenolic glycosides may interact with protein to influence gypsy moth and forest tent caterpillar performance via sev-

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eral means. In response to low levels of nitrogen, foliage-feeding Lepidoptera often increase consumption (Mattson 1980), thereby increasing exposure to allelochemicals (Stamp 1990, Slansky and Wheeler 1992a). Salicortin and tremulacin may be metabolized to quinone methide, phenolic, or orthoquinone products (Clausen et al. 1990, Thompson 1996) which have the potential to induce oxidative stress or bind covalently with proteins, possibly hindering enzymatic reactions and nutrient uptake (Felton et al. 1992, Appel 1993, Summers and Felton 1994). Finally, midgut detoxification enzymes may affect susceptibility of insects to phenolic glycosides (Lindroth and Hemming 1990, Lindroth and Bloomer 1991, Lindroth and Weisbrod 1991, Thompson 1996), and protein-limited insects may experience impaired synthesis of enzymes or conjugation factors.

To address the independent and interactive effects of phenolic glycosides and protein on gypsy moth and forest tent caterpillars, we reared larvae on aspen leaves supplemented with phenolic glycosides and casein and measured indices of insect performance. In addition, we measured activities of midgut enzymes that are likely involved in phenolic glycoside metabolism. These included β -glucosidase, esterase, and glutathione transferase (Lindroth and Hemming 1990, Lindroth and Bloomer 1991, Lindroth and Weisbrod 1991, Thompson 1996). We expected performance of both insect species to be negatively related to phenolic glycoside levels, but more strongly so for gypsy moths. We expected performance of both species to be related positively to protein levels, but more strongly so for forest tent caterpillars. Finally, we expected both species to tolerate higher levels of phenolic glycosides on protein-supplemented leaves.

Materials and Methods

Effects of Phenolic Glycoside- and Protein-Supplemented Leaves on Insect Performance. The experimental design was a 3×2 factorial, with insects reared on diets containing one of three phenolic glycoside concentrations and one of two protein concentrations. Gypsy moth egg masses were provided by USDA-APHIS, Otis Air National Guard Base, MA; and forest tent caterpillar egg bands were collected near Saint Rosaire, Quebec, Canada by Y. Maufette. Before the bioassays, larvae were reared on leaves collected from aspen trees in the University of Wisconsin Arboretum. Larvae were reared at 23°C and a photoperiod of 15:9 (L:D) h. Foliage was sterilized by soaking in a 1% bleach solution for 5 min and then rinsed with water before feeding larvae.

Aspen leaves collected from a single tree in the University of Wisconsin Arboretum were used in the supplementation study. To maintain leaf turgor, the petiole of an individual leaf was inserted through a hole in the top of a 2-ml microcentrifuge tube containing water. The leaves were supplemented with phenolic glycosides partially purified from aspen, as described by Lindroth et al. (1986). High-performance liquid chromatography analysis indicated that

the semipurified phenolic glycoside mixture contained 80% salicortin and tremulacin. The mixture was dissolved in methanol (0.25 g/ml methanol, and 0.5 g/ml methanol for the 2 and 4% stock solutions, respectively) and applied to the top sides of leaves at 0.1 ml/g fresh leaf weight to obtain phenolic glycoside concentrations of 2 and 4% fresh leaf weight. Methanol alone was added to leaves as a control (0% phenolic glycoside) treatment. To supplement leaves with protein, 0.125 g of casein was dissolved per milliliter of KH_2PO_4 buffer (0.075 M, pH 8.0 with 3 mg/ml methylcellulose). The casein solution was applied to the undersides of leaves at 0.4 ml/g fresh leaf weight to obtain a concentration of 5% fresh leaf weight. Buffer alone was added to leaves as a control (0% casein) treatment. Casein was used as the additional protein source because it is a common protein in artificial diets, is readily available, and has been shown to enhance performance of other insect species (Broadway and Duffey 1988).

To determine actual concentrations of phenolic glycosides and nitrogen in the leaves (reported on a dry weight basis), treated and untreated leaves were flash frozen in liquid nitrogen, freeze-dried, ground with a mortar and pestle, and stored in a -20°C freezer before chemical analyses. Water content of untreated leaves was $72 \pm 0.63\%$ (mean \pm SE). Concentrations of the phenolic glycosides tremulacin and salicortin were determined by high-performance thin-layer chromatography. The average phenolic glycoside concentrations for treated leaves were 0.9 ± 0.11 , 5.5 ± 0.41 , and $8.8 \pm 0.47\%$ (dry weight) for 0, 2, and 4% (fresh weight) supplemented leaves. We determined Kjeldahl nitrogen as an indication of protein (casein is 6.25% nitrogen). Leaf samples were digested in acid (Parkinson and Allen 1975) and nitrogen was quantified using a micro-Nesslerization technique (Lang 1958). Glycine *p*-toluenesulfonic acid (5.665% nitrogen) was used as the standard. For nitrogen, concentrations were $3.1 \pm .06$ and $4.5 \pm .12\%$ (dry weight) for 0 and 5% protein-supplemented leaves. Hereafter, treatments will be described according to the percent phenolic glycoside or protein supplemented to the "fresh" leaf weight.

To evaluate insect performance, we conducted a feeding trial in which a newly molted fourth instar was placed into a petri dish (100 by 15 mm) with an aspen leaf from one of the six phenolic glycoside and protein treatments. Initial dry weights for larvae were estimated from 16 newly molted fourth instars of each species. Initial dry weights for leaves were estimated from 9 to 10 leaves per treatment. Assays were run at 25°C under a photoperiod of 15:9 (L:D) h. Each treatment was replicated nine times for each species. Leaves were replenished as needed (at least every 3 d) to prevent larvae from depleting their food. Newly molted fifth instars were frozen, then larvae, frass, and remaining leaves were dried and weighed. Nutritional indices were calculated from standard formulas (Waldbauer 1968) except that initial rather than average weights were used to calculate relative growth and relative consumption rates (Farrar et al. 1989).

Effects of phenolic glycosides and protein on midgut enzyme activities. To determine whether phenolic glycosides and protein influence detoxication enzyme activities, enzyme preparations were made from insects reared on aspen leaves treated with phenolic glycosides or protein. Insects were reared on untreated aspen foliage until the end of the fourth stadium. Newly molted fifth instars were fed leaves from one of the six phenolic glycoside (0, 2, 4% wet weight) by protein (0, 5% wet weight casein) treatments for 2–3 d. Four replicate preparations were made for each treatment and species, with six to eight larvae per replicate. Midguts were removed from larvae, rinsed in 0.2 M potassium phosphate buffer (pH 7.8 with 1 mM EDTA), homogenized with 10 strokes in a Tenbroeck (Corning, NY) tissue grinder and then centrifuged at $10,000 \times g$ for 10 min. The supernatant was then flash frozen in liquid nitrogen and stored at -80°C until enzyme assays were conducted. Protein concentrations of the enzyme preparations were determined using the Bradford (1976) protein assay with bovine serum albumin as the standard. β -Glucosidase activities were determined as described by Lindroth (1988). Briefly, each 1 ml of incubation mixture contained homogenate (90–220 μg protein for gypsy moths or 220–495 μg protein for forest tent caterpillars), 50 μmole of the phenolic glycoside salicin, and 0.1 M potassium phosphate buffer (pH 6.2). Solutions were incubated at 35°C for 60 and 30 min for gypsy moths and forest tent caterpillars, respectively. Glucose liberated by hydrolysis of salicin was measured enzymatically (Sigma Diagnostic Kit 315, Sigma, St. Louis, MO).

Esterase activities were determined by the 1-naphthyl acetate assay (Brattsten 1987). Midgut homogenate (containing 0.4–1.2 μg protein) was added to 0.5 M sodium phosphate buffer (pH 7.8 and 8.0 for forest tent caterpillars and gypsy moths, respectively) to 300 μl volume. The reaction began with addition of 200 μl of 1.25 mM 1-naphthyl acetate (50 mM 1-naphthyl acetate dissolved in ethanol, diluted 40-fold with buffer). After 10 min of incubation at 32°C , the reaction was stopped with the addition of 1.5 ml of a solution containing 7.5 mg/ml sodium dodecyl sulfate and 0.2 mg/ml Fast Blue B. Absorbance (A_{600}) values were read and compared with a 1-naphthol standard curve.

Glutathione transferase activities were measured as the conjugation of 1-chloro-2,4-dinitrobenzene (CDNB) with reduced glutathione (Lindroth 1989). Midgut homogenate (30–75 μg of protein) was mixed in 0.1 M potassium phosphate buffer (pH 8.2) to a volume of 925 μl . Fifty microliters of glutathione solution (50 mM glutathione in phosphate buffer) was added and the reaction was initiated by the addition of 25 μl CDNB (40 mM in methyl cellosolve). The increase in absorbance at 340 nm was measured over 30 s against a blank containing everything except the enzyme. Concentrations of CDNB-glutathione were calculated using an extinction coefficient of $9.6 \cdot \text{mM}^{-1} \cdot \text{cm}^{-1}$.

Statistical Analyses. Data for stadium duration, relative growth and consumption rates, food conversion

efficiencies, and enzyme activities were analyzed with two-way analysis of variance (ANOVA) to identify significant phenolic glycoside and protein effects, and phenolic glycoside by protein interactions. Because insect growth and consumption parameters are strongly affected by initial weight, we analyzed absolute growth rates and absolute consumption rates and total consumption with a two-way analysis of covariance (ANCOVA) using initial larval weight as the covariate (Raubenheimer and Simpson 1992). We include relative growth rate and relative consumption rate analyses to allow for comparisons with earlier studies.

Results

Performance of both gypsy moth and forest tent caterpillars was affected by phenolic glycosides and protein added to aspen leaves (Fig. 1). Duration of the fourth larval stadium was slightly increased (16 and 12% for gypsy moth and forest tent caterpillars, respectively) on the 4% phenolic glycoside treatment. Stadium duration was not influenced by protein supplementation for either species. Relative growth rates were reduced when larvae ate leaves treated with phenolic glycosides, and the reduction in growth caused by 4% phenolic glycosides was greater for gypsy moths (27% decrease) than it was for forest tent caterpillars (14% decrease) (Fig. 1). Overall, the addition of protein to the leaf enhanced relative growth rate by 17% for gypsy moths, but tended to reduce relative growth rate (8%) for forest tent caterpillars. Trends for absolute growth rates paralleled those for relative growth rates. Phenolic glycosides and protein did not interactively influence stadium duration or growth rates in either species.

Consumption was not strongly altered by leaf treatments for gypsy moths (Fig. 2). The marginally significant interaction terms for relative consumption rate and absolute consumption rate reflect the trend of decreasing consumption with increasing phenolic glycosides on the low protein diet, and the opposite response to phenolic glycosides on the high protein diet. Total consumption for gypsy moths was not influenced by the treatments. Forest tent caterpillars marginally increased relative consumption rate, absolute consumption rate, and total consumption in response to phenolic glycosides, and reduced relative consumption rate and absolute consumption rate on leaves supplemented with casein.

Approximate digestibility was slightly reduced with increasing phenolic glycosides for gypsy moths, but not for forest tent caterpillars (Fig. 3). Protein supplementation strongly increased the proportion of the food digested by 39% for gypsy moths and by 24% for forest tent caterpillars. For gypsy moths, the phenolic glycoside-induced decrease in approximate digestibility was more pronounced on low protein diets than on high protein diets. Efficiency of conversion of digested food was altered by phenolic glycosides only for forest tent caterpillars, with a 17% decrease for the 4% phenolic glycoside treatment compared with the control.

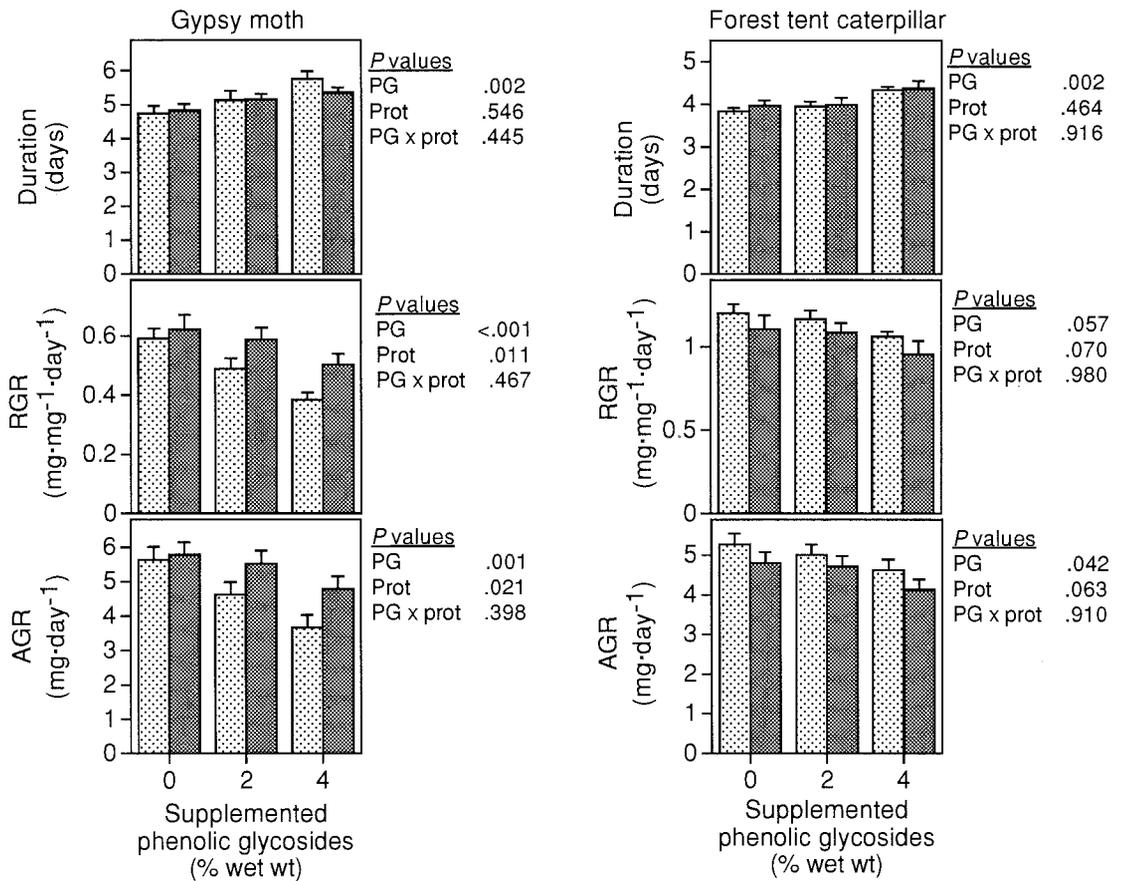


Fig. 1. Effects of phenolic glycoside- and protein-supplemented aspen leaves on gypsy moth and forest tent caterpillar stadia duration and growth rates (RGR, relative growth rate; AGR, absolute growth rates). Light bars represent 0% casein addition, dark bars represent 5% casein addition. *P* values represent the significance of main and interactive effects from ANOVA for duration and relative growth rate, and from ANCOVA for absolute growth rate. Bars represent means \pm 1 SE.

Protein supplementation moderately decreased efficiency of conversion of digested foods for both species. The efficiency of conversion of ingested food for both species decreased with increased phenolic glycosides. Only gypsy moth efficiency of conversion of ingested foods increased on protein-supplemented diets. No interactive effects were found for efficiency of conversion of digested food or efficiency of conversion of ingested food for either species.

Dietary phenolic glycosides, but not protein, influenced gypsy moth enzyme activities (Fig. 4). Increased levels of phenolic glycosides led to a decline in β -glucosidase activities, but an increase in esterase and glutathione transferase activities. For forest tent caterpillars, β -glucosidase activities decreased in response to both additional phenolic glycosides and protein. Esterase activities did not exhibit a consistent change in activity as a result of the dietary treatment, whereas glutathione transferase activities increased with increased levels of phenolic glycosides.

Discussion

Phenolic glycosides have consistently been shown to reduce the performance of gypsy moth and forest tent caterpillars (Lindroth and Hemming 1990, Lindroth and Bloomer 1991, Lindroth and Weisbrod 1991, Hemming and Lindroth 1995, Thompson 1996, Hwang and Lindroth 1997). Results from this study indicate that gypsy moths are generally more susceptible to phenolic glycosides than are forest tent caterpillars, because development times and growth rates were more strongly affected in the former than in the latter.

Supplementation of diets with casein enhanced gypsy moth performance but slightly reduced forest tent caterpillar performance. Although protein is generally considered the nutrient most limiting to leaf-eating Lepidoptera (Mattson 1980), and many examples illustrate that lepidopteran performance is enhanced on high protein diets (Lindroth et al. 1990, Lindroth and Bloomer 1991, Stockhoff 1993, Lindroth et al. 1997), too much protein can stress insects (Schr-

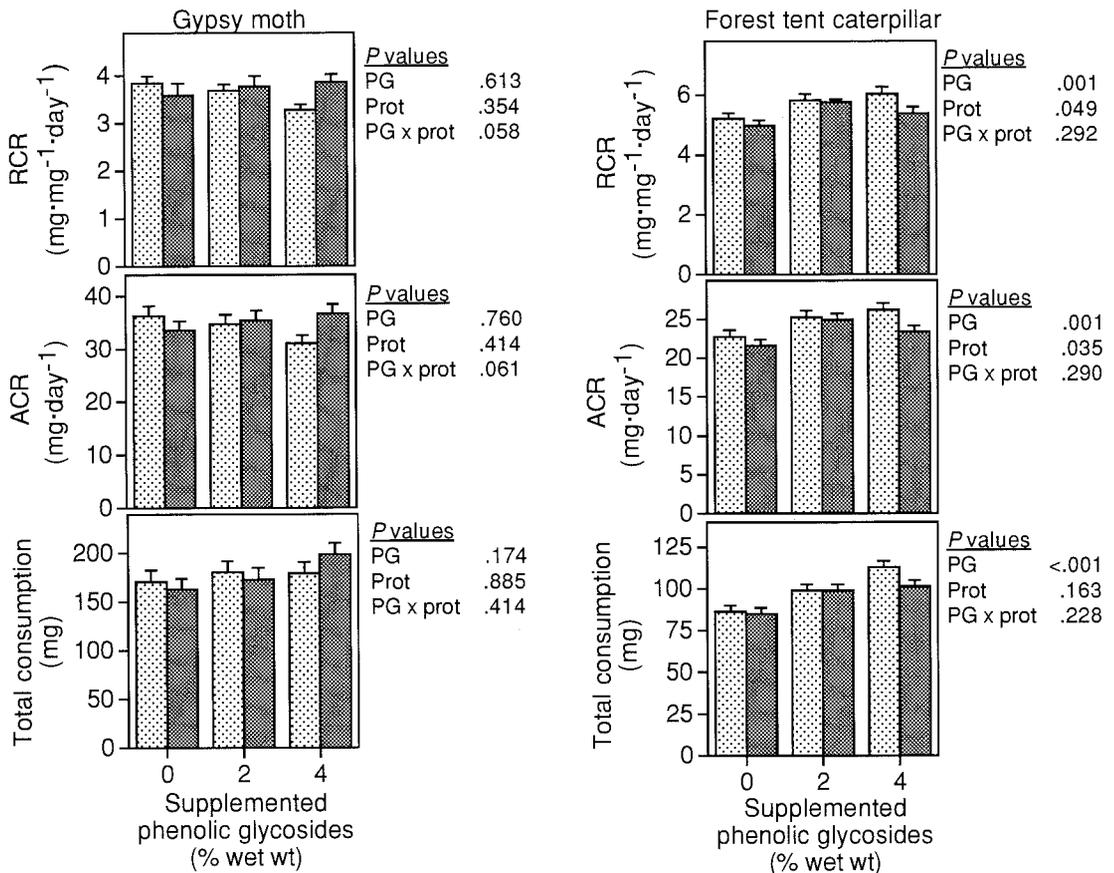


Fig. 2. Effects of phenolic glycoside- and protein-supplemented aspen leaves on gypsy moth and forest tent caterpillar consumption (RCR, relative consumption rate; ACR, absolute consumption rate; consumption is the total consumption). Light bars represent 0% casein addition, dark bars represent 5% casein addition. *P* values represent the significance of main and interactive effects from ANOVA relative consumption rate, and from ANCOVA for absolute consumption rate and total consumption. Bars represent means \pm 1 SE.

oeder 1986, Sheppard and Friedman 1992, Stockhoff 1993). Stockhoff (1993) varied casein in artificial diets fed to gypsy moths and found a curvilinear response to nitrogen. Growth rates increased with increasing nitrogen up to \approx 4% (dry weight) and then fell markedly. The causes of the decreased growth were a drop in consumption rates and leveling off or decline in food conversion efficiencies. In the current study, forest tent caterpillars also decreased consumption rates on high protein diets, and efficiency of conversion of digested foods were strongly reduced on protein-supplemented leaves. The reduced efficiency of conversion of digested foods may indicate a higher metabolic cost associated with nitrogen excretion on diets with excessive protein (Schroeder 1986, Joseph et al. 1993). Gypsy moths were from a lab strain reared for many generations on casein-enriched artificial diets. In contrast, forest tent caterpillars were field collected and therefore unaccustomed to the amino acid composition of casein. As documented for *Anticarsia gemmatalis* (Hübner) by Slansky and Wheeler (1992b), differences in preconditioning may account

for the differential ability of these two species to thrive on high protein diets. Additional research with endogenously low protein foliage supplemented with protein isolated from aspen leaves would provide more useful information.

Phenolic glycosides and protein did not interactively influence insect performance in terms of stadium duration or growth rates. For gypsy moths in particular, the effects were additive. Thus, gypsy moths tolerated higher levels of phenolic glycosides on the casein-supplemented leaves (e.g., larval growth rates on high protein diets with 4% phenolic glycosides were similar to growth rates on low protein diets with 2% phenolic glycosides). The enhanced tolerance was not simply a result of a reduction in exposure to allelochemicals because gypsy moths did not decrease consumption of casein-supplemented leaves when phenolic glycosides were added.

Midgut enzymes are likely important in metabolizing phenolic glycosides (Lindroth and Hemming 1990, Lindroth and Bloomer 1991, Lindroth and Weisbrod 1991, Thompson 1996). By cleaving glucose, metabo-

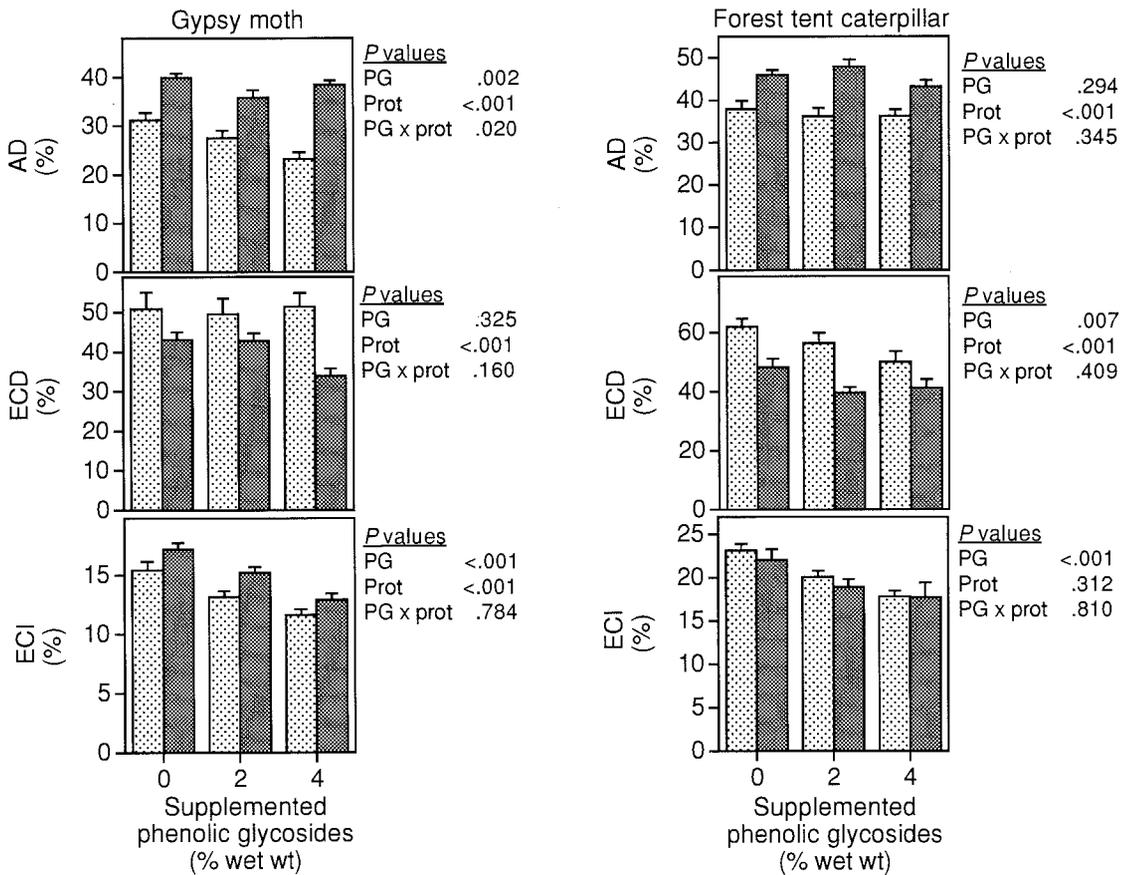


Fig. 3. Effects of phenolic glycoside- and protein-supplemented aspen leaves on gypsy moth and forest tent caterpillar approximate digestibility (AD), efficiency of conversion of digested food (ECD) and efficiency of conversion of ingested food (ECI). Light bars represent 0% casein addition, dark bars represent 5% casein addition. *P* values represent the significance of main and interactive effects from ANOVA. Bars represent means ± 1 SE.

lism of phenolic glycosides by β -glucosidases may bioactivate the compounds, producing toxic aglycones or especially reactive orthoquinone methides (Clausen et al. 1990, Thompson 1996). Supplementing leaf diets with phenolic glycosides led to a decrease in β -glucosidase activity for both gypsy moths and forest tent caterpillars, results consistent with those reported by Lindroth and Hemming (1990). Decreases in β -glucosidase activity may serve to protect larvae from reactive phenolic glycoside metabolites (Clausen et al. 1990, Thompson 1996).

Esterases are thought to detoxify phenolic glycosides because concomitant consumption of phenolic glycosides with an esterase inhibitor potentiates phenolic glycoside toxicity (Lindroth and Hemming 1990, Lindroth and Bloomer 1991, Thompson 1996). Esterase activities are often induced in response to phenolic glycosides (Lindroth and Weisbrod 1991, Lindroth et al. 1993b, but see Lindroth and Hemming 1990), but only gypsy moths exhibited elevated activities in this study. Lindroth and Bloomer (1991) found esterase activities of forest tent caterpillars to be induced by phenolic glycosides when reared on low protein but

not on high protein diets. Results from our current study are consistent with those from the earlier study, because protein (nitrogen) levels in even our non-supplemented diets were moderately high.

Glutathione transferase activities in both species were elevated in response to phenolic glycosides. If phenolic glycosides are metabolized to compounds (e.g., catechol and saligenin) that cause oxidative stress, glutathione transferase could afford protection against them (Thompson 1996). In previous work with artificial diets, however, phenolic glycosides did not induce glutathione transferase activities for gypsy moths (Lindroth and Hemming 1990) or forest tent caterpillars (Lindroth and Bloomer 1991). Because glutathione transferase activities are higher in larvae reared on artificial diets than in insects reared on foliage, further induction might not occur.

Overall, protein levels had little influence on enzyme activities. We expected that increased dietary protein would allow for greater synthesis of detoxication enzymes, and other studies have shown that high protein diets increase activities of some or all of these enzymes (Lindroth et al. 1990, Lindroth and

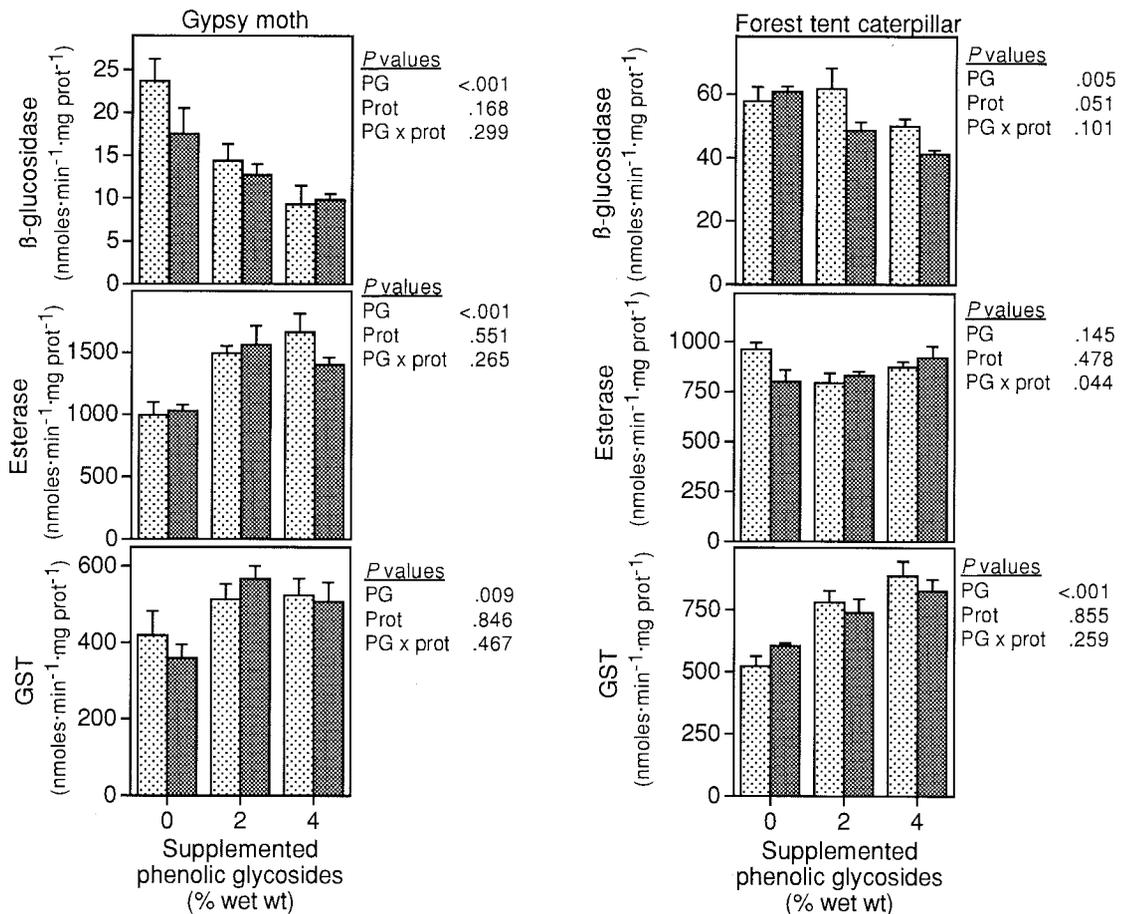


Fig. 4. Effects of phenolic glycoside- and protein-supplemented aspen leaves on gypsy moth and forest tent caterpillar fifth instar midgut enzyme activities (GST, glutathione transferase). Light bars represent 0% casein addition and dark bars represent 5% casein addition. *P* values represent the significance of main and interactive effects from ANOVA. Bars represent means \pm 1 SE.

Bloomer 1991). Given that foliar nitrogen levels were fairly high, adding more protein (especially such a relatively high quality protein as casein) possibly overwhelmed the ability of insects to use the nitrogen. Because protein levels of leaves did not affect enzyme activities, tolerance of phenolic glycosides was not altered by detoxication capacities.

In conclusion, our expectation that performance of both insects species would be reduced by phenolic glycosides, and more strongly so for gypsy moths, was fulfilled. Although we expected insect performance to be enhanced on protein-supplemented leaves, such was the case only for gypsy moths. Contrary to predictions, performance of forest tent caterpillars was reduced on protein-supplemented leaves. Theoretically, high-protein diets could increase the tolerance of insects to phenolic glycosides, but our findings do not support this contention. The effects of phenolic glycosides and protein on gypsy moths were additive, and high protein diets did not cause a decrease in consumption, or an increase in detoxication activities, that could alter susceptibility to phenolic glycosides.

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