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ANTIOXIDANTS IN THE MIDGUT FLUIDS OF A TANNIN-TOLERANT AND A TANNIN-SENSITIVE CATERPILLAR: EFFECTS OF SEASONAL CHANGES IN TREE LEAVES

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Abstract—The seasonal decline in foliar nutritional quality in deciduous trees also effects the availability of essential micronutrients, such as ascorbate and α-tocopherol, to herbivorous insects. This study first examined whether there are consistent patterns of seasonal change in antioxidant concentrations in deciduous tree leaves. α-Tocopherol concentrations increased substantially through time in late summer in sugar maple (*Acer saccharum*), red oak (*Quercus rubra*), and trembling aspen (*Populus tremuloides*). However, seasonal change in the concentrations of other antioxidants differed between each species: *P. tremuloides* had higher levels of ascorbate and glutathione in the spring, *Q. rubra* had higher levels of glutathione but lower levels of ascorbate in the spring, and *A. saccharum* had lower levels of both ascorbate and glutathione in the spring. To test the hypothesis that tannin-tolerant caterpillars maintain higher concentrations of antioxidants in their midgut fluids than do tannin-sensitive species, we measured antioxidants in *Orgyia leucostigma* (a spring- and summer-feeding, tannin-tolerant species) and *Malacosoma disstria* (a spring-feeding, tannin-sensitive species) that were fed tree leaves in the spring and summer. The midgut fluids of *O. leucostigma* larvae generally had higher concentrations of antioxidants in the summer than did those of *M. disstria*, and were significantly higher overall. The results of this study are consistent with the hypothesis that higher concentrations of antioxidants form an important component of the defenses of herbivores that feed on

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mature, phenol-rich tree leaves. Some limitations of the interpretation of total antioxidant capacity are also discussed.

Key Words—Ascorbic acid, α-tocopherol, glutathione, Lepidoptera, herbivore, antioxidant, tree leaves.

INTRODUCTION

Seasonal changes in foliar chemistry are well known. Numerous studies have established that the nutritional quality of tree leaves declines as they mature: protein nitrogen, sugars, phosphorus, and water decrease, while fiber, toughness, and condensed tannins increase (Feeny, 1968, 1970; Scriber, 1977; Ricklefs and Matthew, 1982; Schroeder, 1986; Ayres and MacLean, 1987; Lindroth et al., 1987; Mauffette and Oechel, 1989; Hunter and Lechowicz, 1992). Little work has been done on the seasonal changes in the levels of the three most important low-molecular-weight antioxidants in foliage, i.e., ascorbate (vitamin C), α-tocopherol (vitamin E), and the tripeptide glutathione. The levels of these substances may have important effects on the nutritive value of foliage to herbivorous insects, not only because ascorbate and α-tocopherol are essential micronutrients (Vanderzant et al., 1962; Dadd, 1973; Navon, 1978; Kramer and Seib, 1982; Meister, 1992), but also because they affect oxidative processes that occur in the gut lumens of herbivorous insects. It is well established that the oxidation of potential prooxidants (e.g., many phenolic compounds) in the gut lumens of caterpillars can produce reactive oxygen species and quinones, which can cause oxidative stress and reduce the availability of essential nutrients (Felton et al., 1989; Summers and Felton, 1994; Bi and Felton, 1995; Pardini, 1995; Thiboldeaux et al., 1998; Halliwell and Gutteridge, 1999). Antioxidants enable herbivorous insects to protect themselves from the potential effects of allelochemical oxidation in their gut lumens (Barbehenn et al., 2001) and tissues (Felton et al., 1989; Felton and Duffey, 1992; Summers and Felton, 1994; Bi and Felton, 1995; Timmerman et al., 1999). Accordingly, increased levels of antioxidants improve the fitness of herbivorous insects when they ingest potential prooxidants (Aucoin et al., 1990), and low levels of ingested antioxidants can reduce the fitness of herbivorous insects (Navon, 1978; Lindroth et al., 1991; Felton and Summers, 1993; Lindroth and Weiss, 1994), and make them more susceptible to infection by pathogens (Lindroth et al., 1991).

Malacosoma disstria Hübner (Lasiocampidae), the forest tent caterpillar, and *Orgyia leucostigma* Smith (Lymantriidae), the white-marked tussock moth, are both polyphagous Lepidoptera that feed on the three tree species examined in this study (Stehr and Cook, 1968; Baker, 1972; Nicol et al., 1997; Panzuto et al., 2001). However, they have different life-history strategies. While *M. disstria* has a single generation of larvae in the spring, *O. leucostigma* has two or more generations that feed either on young foliage in the spring or on fully mature leaves in the summer. While *O. leucostigma* larvae are unaffected by the consumption of diets containing

as much as 8% (dry weight) tannic acid, as little as 0.5% tannic acid causes lethal pupal deformities in *M. disstria* (Karowe, 1989). Higher concentrations of antioxidants in the midgut fluid of *O. leucostigma* are associated with its ability to tolerate ingested tannins by limiting phenol oxidation (Barbehenn and Martin, 1992; Barbehenn et al., 2001). In contrast, extensive phenol oxidation occurs in the midgut lumen of *M. disstria* (Barbehenn and Martin, 1994; Barbehenn et al., 2001, 2003). In this study, we tested the hypothesis that *O. leucostigma* maintains higher concentrations of antioxidants in its midgut fluid than does *M. disstria* when they feed on tree leaves, especially in the summer, when elevated concentrations of phenols are commonly present in deciduous tree leaves.

If *M. disstria* larvae are poorer at maintaining antioxidants in their midgut fluids than are larvae of *O. leucostigma* when feeding on artificial diet, we expected that *M. disstria* might rely on ingesting higher concentrations of antioxidants in spring foliage. The small number of studies that have explored seasonal variation in the levels of antioxidants in tree foliage show few consistent patterns between tree species and antioxidants (Dash and Jenness, 1985; Kunert and Ederer, 1985; Schupp and Rennenberg, 1988; Luwe, 1996). Therefore, an initial goal of this study was to establish the concentrations of the three major antioxidants and total antioxidant capacity (TAC) in the leaves of three host tree species. Ascorbic acid, glutathione, α-tocopherol, and TAC were measured in trembling aspen (*Populus tremuloides* Michaux), sugar maple (*Acer saccharum* Marshall), and red oak (*Quercus rubra* L.) in the spring and summer. Antioxidant levels and TAC were also compared in the midgut fluids of *O. leucostigma* and *M. disstria* when they fed on the leaves of these trees in the spring and summer. The findings allowed us to compare previous results obtained with artificial diets with those obtained with host plant foliage.

METHODS AND MATERIALS

Trees. Six trees of *P. tremuloides*, *Q. rubra*, and *A. saccharum* were tagged for study, including three each from two sites, the Matthaei Botanical Gardens and Keubler–Langford Park (Ann Arbor, MI). These sites are over 10 km apart and are separated by the Huron River. Selected trees were growing in the open or at the edge of forests, providing foliage that was exposed to full sun. Trees were estimated to be approximately 30–50 years old, based on their trunk diameters. Twigs with several leaves (2–4 m high) were cut from each tree using a pole pruner. Leaves with minimal damage from insects and pathogens were chosen from each tree. Samples were placed in plastic bags and stored on ice in a cooler immediately upon collection (10:00–11:30 AM). These leaves were either fed to caterpillars or were extracted during a 3-hr period in the afternoon. Trees were sampled on May 11 (spring), July 13 (summer), and August 24 (late summer) in 2000. Spring foliage was still soft and light green, but appeared to be fully expanded.

The second leaf from the tip of a twig was chosen for chemical analysis. Samples ($N = 6$ per species) were processed in a random order with respect to species and site. The petiole and midrib were removed with a razor blade, and each of the two leaf halves were weighed (± 0.1 mg) and processed separately. Leaf samples were placed in a chilled mortar and ground to a fine powder in liquid N_2. The leaf powder was mixed into a slurry with additional liquid N_2 and poured into a chilled centrifuge tube (15 ml, Corning; plug-seal cap) containing either N_2-purged ethanol (3.0 ml) or 5% (w/v) metaphosphoric acid (3.0 ml, containing 1 mM EDTA). Ethanol extracts were flushed with N_2, and all extracts were placed in a shaker (30 min, 4°C). After centrifugation ($1{,}000 \times g$, 4°C), an aliquot (1.0 ml) of each ethanol extract was mixed with 10 μl of butylated hydroxytoluene in ethanol (4 mM final concentration) in a microcentrifuge tube and flushed with N_2. All samples were stored at -75°C until analyzed. At the time of leaf extraction, a second leaf (matched for twig position and appearance) was weighed fresh (after removing its midrib and petiole), and reweighed after drying at 60 or 70°C for 4 days to determine the percent water in the leaves ($N = 6$ per species).

Insects and Midgut Fluid Collection. Eggs of *O. leucostigma* and *M. disstria* were obtained from the Canadian Forest Pest Management Institute (Sault Ste. Marie, Ontario), and larvae were reared as described previously (Barbehenn et al., 2001). Final-instar larvae were placed at random in plastic shoe boxes containing leaves from *P. tremuloides*, *Q. rubra*, or *A. saccharum*. Leaves from the same two (tagged) trees of each species were used for feeding larvae of both species in the spring and summer, and these leaves were collected from the same region of the trees at the same time of the day as were the leaves collected for antioxidant analyses. Leaves were kept hydrated by placing their twigs in water in 15-ml tubes. Insects were fed for 2–2.5 days, with fresh leaves being provided daily. Larvae were dissected on the third day: *M. disstria* on May 12 and July 12, and *O. leucostigma* on May 17 and July 5. Dissections were performed between 1 and 5 hr after larvae were placed on fresh leaves, as described previously (Barbehenn et al., 2001). The midgut contents were placed immediately in tared 2-ml microcentrifuge tubes containing 300 or 350 μl of N_2-purged (1 min/ml) double-distilled water, and the headspace in each tube was flushed with N_2. The fresh weight of the sample was recorded. Gut contents were vigorously shaken and centrifuged ($8{,}000 \times g$, 5 min, ambient temperature). Aliquots (100 μl) of the supernatant solutions were placed in 400 μl of 5% MPA with 1 mM EDTA for ascorbate and glutathione analysis. Aliquots (50 μl) of the supernatant solutions were placed in 200 μl of N_2-purged ethanol for measuring TAC. Aliquots (100 μl) of the supernatant solutions were placed in 400 μl of N_2-purged ethanol with BHT (4 mM final concentration) for α-tocopherol analysis. The headspace above each ethanolic extract was flushed with N_2, and all extracts were stored at -75°C until analyzed. An average of eight midgut samples per tree species (range = 5–12) were taken on each date. The fresh

weights of gut contents were converted to volumes based on the basis of percent water in similar samples ($N = 2$–3/species). Samples for fresh weight/dry weight (FW/DW) ratios were reweighed after drying (70°C, 2 days).

Antioxidant Analysis. Ascorbate, glutathione, and α-tocopherol were measured using high-performance liquid chromatography, as described previously (Barbehenn, 2003). Ascorbate was analyzed within 1–4 months, total glutathione (GSH + 2×GSSG) within 7–11 months, and α-tocopherol within 5–9 months from the time of storage. Antioxidant stability was examined in a previous study (Barbehenn, 2003), but was tested further for ascorbate in this study. Ascorbate remained at 90–95% of initial levels in leaf extracts from the spring and summer, and in gut fluids of *M. disstria* (fed the spring foliage of *A. saccharum*; $N = 4$) after 11–12 months of storage. However, ascorbate measured in the gut fluids of *O. leucostigma* (fed the spring foliage of *A. saccharum*; $N = 4$) was at 42% of the original levels measured after 11–12 months of storage, suggesting that ascorbate levels may have been diminished to some extent in the original measurements.

TAC was estimated in ethanol extracts of leaves and midgut fluids by measuring their ability to reduce 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\cdot+}$) compared with Trolox standards (Re et al., 1999), as described previously (Barbehenn, 2003). Samples were analyzed within 0.5–2.5 months from the time of storage, times within which they should remain stable (Barbehenn, 2003). Ethanol extracts do not include glutathione because of its low solubility in ethanol (personal observation), but do include ascorbate, lipophilic antioxidants, and most of the phenolic compounds present in tree leaves (Swain, 1979; Pedersen, 2000).

Comparison of Antioxidant Concentrations. Antioxidant concentrations in tree leaves were compared on a moles per gram fresh weight (FW) basis. This measure incorporates seasonal changes in antioxidant concentration in the liquid fraction of the leaf, as well as changes in water content. Concentrations of antioxidants in midgut fluids were examined on a molar basis. Analyses of antioxidant concentrations and percent water were made with SAS version 8e, using PROC MIXED without a random variable (SAS, 2000). Models included tree species and season as fixed factors, along with the species × season interaction, to compare foliar antioxidant concentrations. Season was used as a repeated measure. Antioxidant concentrations in midgut fluid were compared using models that included insect species, tree species, and season as fixed effects, and included all two-way and three-way interactions. Paired comparisons of all means (presented in all figures and tables) were made using the differences of least-squares means, calculated with PROC MIXED (SAS, 2000). Antioxidant concentrations in gut fluids were log-transformed to fit a normal distribution. The overall difference between *O. leucostigma* and *M. disstria* was determined with a chi-square test (Sokal and Rohlf, 1981). The means of 24 independent measurements of antioxidant concentrations in each insect species were contrasted pairwise, and the observed number of cases

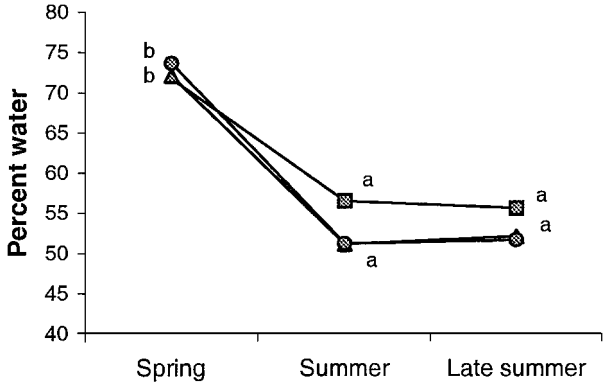

FIG. 1. Percent water in the leaves of *Populus tremuloides* (triangle), *Quercus rubra* (circle), and *Acer saccharum* (square). Different letters denote significant differences between means throughout the entire figure ($P < 0.05$). Standard error bars are smaller than the symbols.

in which each species had a higher antioxidant concentration was compared to an expected distribution of 12 cases for each species.

RESULTS

Seasonal Changes in Foliar Antioxidant Concentrations. The water content of the leaves of each species changed in a similar pattern between spring and summer (Figure 1), suggesting that differences in antioxidant concentrations between species are not due to differences in FW/DW ratios. Only season produced a significant effect ($P < 0.001$), though tree species and the season × tree species interaction showed a trend toward significance ($P = 0.066$ and 0.071, respectively).

Ascorbate. Ascorbate levels declined by 51% in *P. tremuloides* from the spring to the late summer ($P < 0.001$) (Figure 2A). In comparison, *Q. rubra* and *A. saccharum* leaves contained their lowest levels of ascorbate in the spring, and showed a trend toward increasing levels during the summer. Levels of ascorbate in *A. saccharum* increased by 117% and in *Q. rubra* by 28% in the late summer (Figure 2A), though these were not statistically significant changes in this study ($P = 0.118$ and 0.432, respectively). Significant effects included tree species ($P < 0.001$) and the season × species interaction ($P < 0.001$).

Glutathione. Glutathione decreased by 53% from the spring to the late summer in *P. tremuloides* ($P < 0.001$) and decreased by 55% in *Q. rubra* ($P = 0.012$) (Figure 2B). By contrast, glutathione increased by 60% in *A. saccharum* from a relatively low level in the spring ($P = 0.011$). An average of over 65% of the total glutathione measured was in the reduced form [calculated as GSH/(GSH + 2×GSSG) × 100]. Total glutathione concentrations (GSH + 2×GSSG) reported

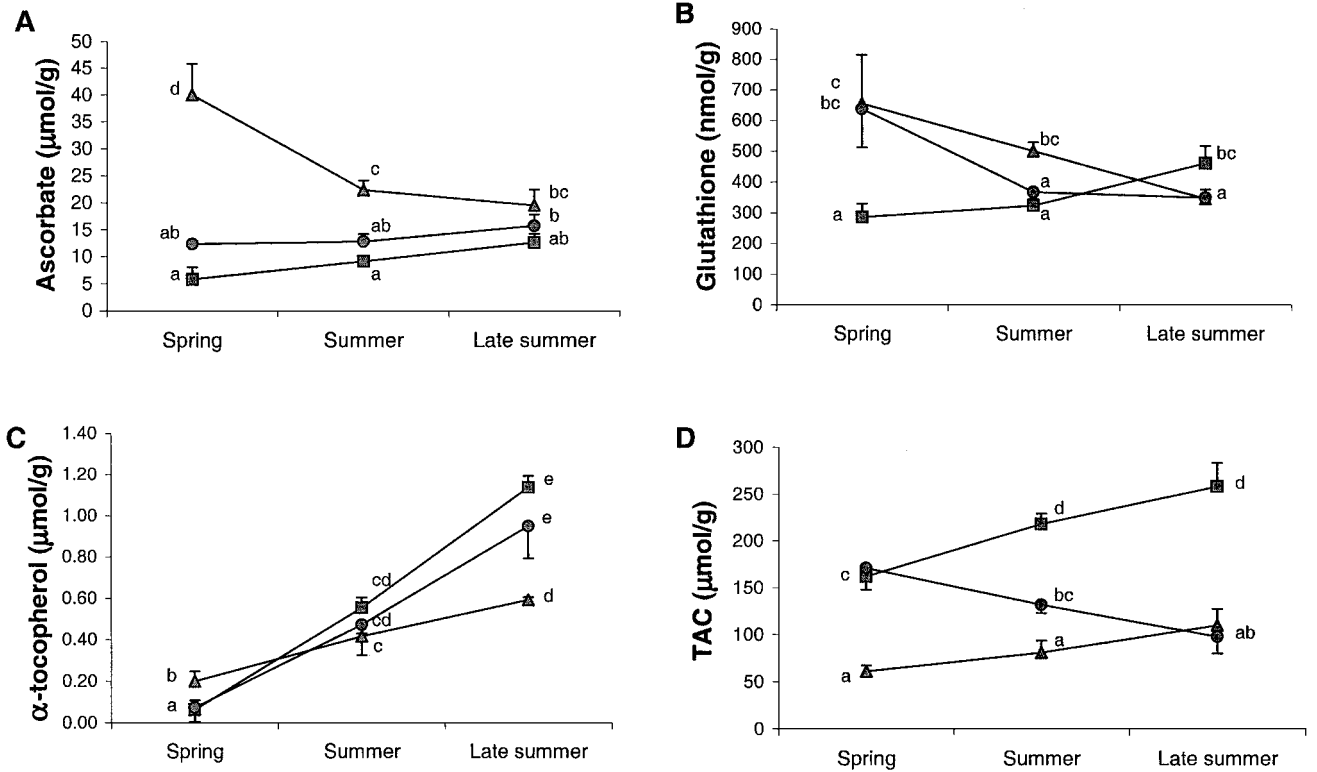

FIG. 2. (A) Ascorbate in the leaves of *Populus tremuloides* (triangle), *Quercus rubra* (circle), and *Acer saccharum* (square). (B) Total glutathione (GSH + 2×GSSG). (C) α-Tocopherol. (D) Total antioxidant capacity (TAC). Concentrations are on a fresh weight basis. Non-overlapping letters denote significant differences between means throughout the entire figure ($P < 0.05$). Standard error bars are presented.

in this study include GSSG that was present in the original samples and that which was formed during sample preparation. Therefore, the fraction of reduced GSH measured by this method is an underestimate. Significant effects included tree species ($P = 0.011$), season ($P = 0.039$), and the interaction between season and species ($P < 0.001$).

α-Tocopherol. α-Tocopherol levels increased substantially in each tree species through the spring and late summer (Figure 2C). Levels increased by 3-fold in *P. tremuloides*, 13-fold in *Q. rubra*, and 19-fold in *A. saccharum* ($P < 0.001$ in each species). *P. tremuloides* foliage contains significantly higher α-tocopherol levels in the spring than the other tree species ($P = 0.004$), but is surpassed by *A. saccharum* and *Q. rubra* by late summer. Significant effects included season ($P < 0.001$), tree ($P = 0.035$), and season × species interaction ($P < 0.001$).

Total Antioxidant Capacity. TAC in the spring foliage of *A. saccharum* and *Q. rubra* was similar ($P = 0.959$), and 166–180% higher than that in *P. tremuloides* ($P < 0.001$) (Figure 2D). In the summer, TAC showed an upward trend in *P. tremuloides* ($P = 0.055$) and increased by 59% in *A. saccharum* ($P = 0.010$), but decreased by 42% in *Q. rubra* by late summer ($P = 0.078$). Significant effects included tree species ($P < 0.001$) and the season × species interaction ($P = 0.002$).

Antioxidant Concentrations in Caterpillars. The water content of midgut fluids was maintained in the range of 88–93% when *O. leucostigma* and *M. disstria* fed on tree leaves of each species, despite differences in the water content of the foliage ingested in the spring and summer. Therefore, seasonal differences in antioxidant concentrations in gut fluids were not affected by changes in their water content.

Ascorbate. O. leucostigma maintained higher ascorbate levels in the summer than did *M. disstria* ($P = 0.002$), but the levels in the two species were not significantly different in the spring ($P = 0.823$) (Table 1). Levels of ascorbate in both species were substantially lower in the summer than in the spring ($P < 0.001$). In several cases, it is evident that foliar ascorbate concentration was positively associated with its concentration in *M. disstria* and *O. leucostigma*. For example, the

TABLE 1. ASCORBATE CONCENTRATIONS (μM) IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* WHEN FEEDING ON TREE FOLIAGE IN THE SPRING AND SUMMER

	Spring		Summer	
Tree species	*M. disstria*	*O. leucostigma*	*M. disstria*	*O. leucostigma*
Populus tremuloides	910 ± 328^{d} (8)	$1{,}589 \pm 632^{d}$ (9)	12 ± 4^{ab} (7)	68 ± 29^{bc} (12)
Acer saccharum	70 ± 16^{c} (8)	66 ± 10^{bc} (7)	27 ± 10^{bc} (6)	178 ± 103^{c} (11)
Quercus rubra	334 ± 55^{d} (8)	555 ± 121^{d} (9)	6 ± 3^{a} (5)	24 ± 6^{bc} (8)

Note: Data are presented as mean ± SE (N). Non-overlapping following SEs letters designate significantly different means across the entire table ($P < 0.05$).

low ascorbate level in *A. saccharum* in the spring is associated with low ascorbate levels in the gut fluids of both caterpillar species, and high ascorbate levels in *P. tremuloides* is associated with high levels in the gut fluids of both caterpillar species (Table 1). It is also evident that foliar ascorbate levels in the summer had little relationship to the concentrations present in the midgut fluids of the insect species at this time (Table 1). Other significant effects included insect species ($P = 0.017$), season ($P < 0.001$), and interactions between insect species and season ($P = 0.037$) and tree species and season ($P < 0.001$).

Glutathione. O. leucostigma midgut fluids contained higher concentrations of glutathione than did those of *M. disstria* in the spring ($P < 0.001$), but *M. disstria* contained higher glutathione concentrations than did *O. leucostigma* in the summer ($P < 0.001$) (Table 2). While concentrations in *O. leucostigma* decreased in the summer to as little as 5.5% of spring levels ($P < 0.001$), glutathione concentrations in *M. disstria* increased in the summer ($P = 0.009$), primarily when feeding on *A. saccharum* (Table 2). Decreases in *O. leucostigma* are partially attributable to decreases in the glutathione levels in the foliage, which dropped to 79% and 58% of spring levels in *P. tremuloides* and *Q. rubra*, respectively (Figure 2b). The total glutathione in midgut fluid contained over 66% and 52% reduced glutathione in *M. disstria* and *O. leucostigma*, respectively. Significant effects included season ($P = 0.015$) and interactions between insect species and season ($P < 0.001$) and tree species and season ($P < 0.001$).

α-Tocopherol. O. leucostigma midgut fluids contained higher concentrations of α-tocopherol than did those of *M. disstria* in the summer ($P < 0.001$). α-Tocopherol concentrations were not significantly different between the insect species in the spring ($P = 0.299$). In both insect species, α-tocopherol increased in the summer when feeding on *P. tremuloides*, and also increased in *O. leucostigma* feeding on *A. saccharum* (Table 3). α-Tocopherol concentrations were lowest in the midgut fluids of both insect species when feeding on *Q. rubra* in the spring and the summer (Table 3). Significant effects included insect species ($P = 0.001$), tree species ($P < 0.001$), season ($P < 0.001$), and interactions between season and insect species ($P = 0.051$), season and tree species ($P = 0.006$), and insect species and tree species ($P = 0.029$).

Total Antioxidant Capacity. TAC was significantly higher in *O. leucostigma* than in *M. disstria* in the summer ($P < 0.001$), but was similar in the spring between the two caterpillar species ($P = 0.294$) (Table 4). Differences were most evident between the insect species when they fed on *A. saccharum*: TAC in *O. leucostigma* remained relatively high in the summer, whereas TAC in *M. disstria* declined in the summer to a lower level than that found in the spring (Table 4). TAC in the midgut fluids of both caterpillar species was consistently low when they were fed *Q. rubra*, lower than when fed on *P. tremuloides* ($P < 0.001$), which in turn was lower than TAC levels noted when they were fed on *A. saccharum* ($P = 0.017$). Significant effects included insect species ($P < 0.001$), tree species ($P < 0.001$),

TABLE 2. GLUTATHIONE CONCENTRATIONS (μM) IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* WHEN FEEDING ON TREE FOLIAGE IN THE SPRING AND SUMMER

	Spring		Summer	
Tree species	*M. disstria*	*O. leucostigma*	*M. disstria*	*O. leucostigma*
Populus tremuloides	81.1 ± 10.4^{cd} (8)	278.6 ± 117.6^{d} (9)	76.9 ± 8.1^{cd} (7)	23.5 ± 3.5^{a} (12)
Acer saccharum	$29.6 + 2.2^{ab}$ (8)	100.5 ± 20.1^{cd} (9)	222.1 ± 59.3^{d} (6)	61.4 ± 11.1^{bc} (11)
Quercus rubra	34.6 ± 7.5^{ab} (8)	397.1 ± 18.1^{d} (9)	49.8 ± 11.4^{abc} (5)	22.4 ± 6.3^{a} (9)

Note: Data are presented as mean $\pm$ SE (N). Non-overlapping letters following SEs.

TABLE 3. α-TOCOPHEROL CONCENTRATIONS (μM) IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* WHEN FEEDING ON TREE FOLIAGE IN THE SPRING AND SUMMER

	Spring		Summer	
Tree species	*M. disstria*	*O. leucostigma*	*M. disstria*	*O. leucostigma*
Populus tremuloides	10.8 ± 1.1^{a} (8)	13.6 ± 1.6^{a} (9)	30.4 ± 3.2^{bc} (7)	61.7 ± 7.8^{d} (12)
Acer saccharum	$9.0 + 1.0^{a}$ (8)	18.5 ± 4.3^{ab} (9)	9.0 ± 1.2^{a} (6)	42.5 ± 7.8^{c} (11)
Quercus rubra	4.2 ± 0.7^{a} (8)	3.4 ± 0.4^{a} (9)	5.2 ± 1.1^{a} (5)	7.6 ± 1.7^{a} (8)

Note: Data are presented as mean $\pm$ SE (N). Non-overlapping letters following SEs designate significantly different means across the entire table ($P < 0.05$).

and interactions between tree species and season ($P < 0.001$), insect species and season ($P = 0.015$), and season $\times$ insect species $\times$ tree species ($P = 0.002$).

Overall Comparison of Insect Species. When antioxidant concentrations in *O. leucostigma* and *M. disstria* were compared pairwise across all tree species and seasons (24 independent comparisons), the midgut fluid of *O. leucostigma* was found to contain significantly greater antioxidant concentrations than the midgut fluid of *M. disstria* ($\chi^2 = 6.0$; $P < 0.025$).

DISCUSSION

Contrary to our expectation, caterpillars that feed in the spring do not generally encounter higher antioxidant concentrations than they would in the summer. Instead, foliar concentrations of antioxidants change in species-specific patterns. While α-tocopherol concentrations increased substantially through time in the examined tree species, seasonal changes in the concentrations of ascorbate and glutathione differed in each tree species: *P. tremuloides* leaves contained higher levels of ascorbate and glutathione in the spring, *Q. rubra* had lower concentrations

TABLE 4. TOTAL ANTIOXIDANT CAPACITY (TAC) (mM) IN THE MIDGUT FLUIDS OF *Malacosoma disstria* AND *Orgyia leucostigma* WHEN FEEDING ON TREE FOLIAGE IN THE SPRING AND SUMMER

	Spring		Summer	
Tree species	*M. disstria*	*O. leucostigma*	*M. disstria*	*O. leucostigma*
Populus tremuloides	22.1 ± 1.8^{a} (8)	35.0 ± 3.7^{bc} (13)	43.6 ± 7.5^{cd} (7)	54.1 ± 6.3^{d} (12)
Acer saccharum	$62.1 + 9.4^{d}$ (8)	51.4 ± 4.8^{d} (13)	25.7 ± 6.0^{ab} (6)	58.0 ± 5.6^{d} (10)
Quercus rubra	25.4 ± 2.3^{ab} (8)	26.2 ± 2.0^{ab} (13)	21.0 ± 5.0^{a} (5)	24.6 ± 1.7^{ab} (8)

Note: Data are presented as mean $\pm$ SE (N). Non-overlapping letters following SEs designate significantly different means across the entire table ($P < 0.05$).

of ascorbate but higher concentrations of glutathione in the spring, and *A. saccharum* had lower concentrations of both ascorbate and glutathione in the spring. The trend toward increased ascorbate concentration in the mature leaves of *A. saccharum* and *Q. rubra*, though not significant in this study, fits the pattern of seasonal changes observed previously in *Fagus sylvatica* (Luwe, 1996) and *Pinus strobus* (Anderson et al., 1991), while decreases in glutathione, such as in *P. tremuloides* and *Q. rubra*, have also been observed in *F. sylvatica* and some nondeciduous trees (Dash and Jenness, 1985; Kunert and Ederer, 1985; Schupp and Rennenberg, 1988; Luwe, 1996). On the basis of these examples, the elevated levels of ascorbate in the spring foliage of *P. tremuloides* and the low level of glutathione in the spring foliage of *A. saccharum* appear to be exceptions to the more common patterns of seasonal change in deciduous tree leaves.

In general, previous studies on antioxidants in deciduous tree leaves have found concentrations similiar to those measured in this study, e.g., 6–20 μmol ascorbate/g FW (Luwe, 1996; Schwanz et al., 1996a,b; Marabottini et al., 2001; but see also Roth et al., 1994). In contrast, *P. tremuloides* leaves contain an exceptionally high concentration of ascorbate in the spring (40 μmol/g or 2.5% DW). Previously, concentrations of glutathione and α-tocopherol have been measured in the ranges of approximately 200–700 nmol/g FW and 150–2,400 μmol/g FW, respectively (Kunert and Ederer, 1985; Strohm et al., 1995; Luwe, 1996; Schwanz et al., 1996a,b; Marabottini et al., 2001). The pattern of increasing levels of α-tocopherol during the growing season appears to be typical of developing leaves (Kunert and Ederer, 1985; Hess, 1993).

The results of this study support the hypothesis that *O. leucostigma* maintains higher concentrations of antioxidants in its midgut fluids than does *M. disstria* when they feed on tree leaves, as was the case when they feed on artificial diets (Barbehenn et al., 2001). Significantly greater concentrations of ascorbate and α-tocopherol and higher TAC values were present in the midgut fluids of *O. leucostigma* compared to *M. disstria* when they fed on mature tree leaves. However, glutathione showed the opposite pattern in the summer, and we are unable to explain this or other cases in which the relationship between foliar and larval antioxidant concentrations appears to be complex. The concentration of an antioxidant in the midgut fluid would depend on the efficiency of its extraction from leaf tissues and the relative rates of its metabolism, absorption, and/or secretion in the midgut lumen. Several clear cases were apparent, however, which support the idea that caterpillars could increase the antioxidant concentrations in their midgut fluids by feeding on the foliage of particular tree species, especially in the spring. Most notably, *M. disstria* and *O. leucostigma* contain very high concentrations of ascorbate and glutathione when they feed on the spring foliage of *P. tremuloides*.

Although we have not made measurements that demonstrate whether differences in antioxidant concentration affect insect fitness, our observation of browning in midgut fluids is potentially important in this regard. Browning was common in

M. disstria when it fed on each tree species in the summer, but no browning was observed in *O. leucostigma* feeding in the summer, nor in any case when either species fed on spring foliage. Browning is a well-known result of extensive phenol oxidation (Cilliers and Singleton, 1989) and has been observed previously in the gut lumens of *M. disstria* on phenol-containing diets and in some foliage-feeding insects (Barbehenn and Martin, 1994; Appel and Maines, 1995; Barbehenn et al., 1996; Johnson and Felton, 1996). Decreased TAC in the midgut fluids of *M. disstria* in the summer (primarily on *A. saccharum*) is consistent with the possibility that ingested phenols were oxidized in this species (Barbehenn and Martin, 1994; Barbehenn et al., 2001, 2003). Potential consequences of extensive phenol oxidation in the midgut lumen include decreased essential nutrient availability and oxidative stress.

In an earlier study, it was concluded that the presence of a complete ascorbate recycling system (ascorbate, dehydroascorbate reductase, and glutathione) in the midgut lumen can explain the tolerance of *O. leucostigma* to tannins ingested in an artificial diet (Barbehenn et al., 2001). Efficient ascorbate recycling would maintain sufficiently high levels of ascorbate to prevent tannin oxidation, and the low concentration of glutathione in the midgut fluid of *M. disstria* (6.5 μM) would preclude the recycling of ascorbate in this species. However, it is clear that *M. disstria* obtains much larger amounts of glutathione from tree leaves (Table 2). Yet, even in these conditions its midgut fluids contain extremely low levels of ascorbate and exhibit signs of phenol oxidation when feeding on mature foliage. Although levels of glutathione appear relatively low in the summer in *O. leucostigma*, they are strikingly similar to levels that adequately protect them when they feed on an artificial diet (23 μM): 22 μM on *Q. rubra* and 23 μM on *P. tremuloides*. Given the broad range of glutathione concentrations in these foods (10–1000 μM), this apparent homeostasis suggests that *O. leucostigma* actively maintains a minimum glutathione level in the midgut lumen, possibly as a result of glutathione secretion (Barbehenn et al., 2001).

TAC was initially measured in this study as a means of estimating the overall concentration of antioxidants present in the sample extracts. However, the variety of antioxidants that were not directly measured, such as carotenoids and cysteine, appear to make a relatively minor contribution to foliar TAC. The concentrations of other commonly recognized antioxidants in leaves are quite small compared with the TAC of 60–260 μmol/g measured in this study. For example, β-carotene and other carotenoids are found at between 0.5 and 0.8 μmol/g in tree leaves (Schwanz et al., 1996a,b), and cysteine and γ-glutamylcysteine are at only 0.055 and 0.020 μmol/g, respectively (Schwanz et al., 1996b) (our calculations). When the higher Trolox equivalent antioxidant capacity (TEAC) of carotenoids (2.5- to 3-fold greater than Trolox) (Re et al., 1999) is taken into account, the above antioxidants would contribute no more than 0.6–4.1% of the TAC in tree leaves. In comparison, the contribution of ascorbate and α-tocopherol to the TAC of foliar extracts in this study together ranged from a minimum of 5.4% (*A. saccharum*

in the late summer) to a maximum of 65.9% (*P. tremuloides* in the spring). It is likely that foliar phenols contribute most of the undetermined $ABTS^{\cdot+}$ reducing power measured with the TAC assay: (1) TAC is positively correlated with total phenol concentrations in a wide variety of plant species (Pietta et al., 1998; Prior et al. 1998; Velioglu et al., 1998); (2) phenolic compounds are the most abundant "antioxidants" in leaves (Pratt, 1992), reaching high levels (10–25% DW) in the tree species in this study (Ricklefs and Matthew, 1982; Schultz et al., 1982; Lindroth et al., 1987); (3) phenolic compounds have high TEAC values, e.g., up to 5-fold greater than Trolox for low molecular weight phenols (Re et al., 1999), and 15- and 28-fold greater than Trolox for hydrolyzable and condensed tannins, respectively (Hagerman et al., 1998); and (4) seasonal changes in TAC match the patterns observed previously for total phenols, e.g., concentrations of phenolic compounds in *A. saccharum* and *P. tremuloides* increase seasonally (Lindroth et al., 1987; Hagerman, 1988), and concentrations of total phenolic compounds in *Q. rubra* decrease seasonally (Ricklefs and Matthew, 1982; Rossiter et al., 1988; Appel et al., 2001) (Figure 2d). Unless we are unaware of a major source of $ABTS^{\cdot+}$-reducing antioxidants in leaves, the above results suggest that TAC in our study was commonly dominated by phenols.

Phenolic compounds could function either as antioxidants or prooxidants in the caterpillar midgut fluids (Barbehenn et al., 2001; Johnson and Felton, 2001). The in vitro conditions used in the measurement of TAC do not necessarily discriminate between these possibilities. Measurement of the activity of these compounds in the physicochemical conditions present in vivo is necessary to determine their biological function (Strube et al., 1997). For example, midgut fluids containing a combination of high phenol and low ascorbate concentrations would be expected to produce high TAC. However, phenols in the midgut fluids of caterpillars in these conditions would likely act as prooxidants (i.e., form semiquinone radicals). Recent measurements of semiquinone radical intensities are consistent with this interpretation. Although *M. disstria* has high TAC values when feeding on *A. saccharum* in the spring (Table 4), it also contains a relatively low concentration of ascorbate (Table 1), and high levels of semiquinone radicals are generated in its midgut fluids (Barbehenn et al., 2003). In comparison, when *M. disstria* feeds on *Q. rubra* in the spring, low semiquinone radical intensities are generated (Barbehenn et al., 2003), consistent with lower TAC (i.e., phenol concentration) and a higher ascorbate concentration (Tables 1 and 4). An alternative hypothesis, that ingested phenols function as antioxidants (Johnson and Felton, 2001), appears to be most plausible in cases in which phenols are present at low concentrations relative to ascorbate and/or in which phenols may be relatively stable, such as in acidic biological fluids.

Future research on the antioxidant defenses of *M. disstria* and *O. leucostigma* needs to address three issues, including their oviposition and foraging preferences, the effectiveness of specific antioxidants, such as α-tocopherol, in suppressing

phenol oxidation in the midgut, and the effect of variation in antioxidant concentrations on larval performance. The fact that midgut antioxidant concentrations are affected by the species of tree foliage consumed (primarily in the spring) suggests that adult oviposition preference and/or larval foraging could influence their midgut antioxidant systems, e.g., restricting oviposition or consumption to tree species, tree genotypes, or individual leaves containing high concentrations of the most effective antioxidants.

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REFERENCES

ANDERSON, J. V., CHEVONE, B. I., and HESS, J. L. 1991. Seasonal variation in the antioxidant system of eastern white pine needles. *Plant Physiol*. 98:501–508.

APPEL, H. M. and MAINES, L. W. 1995. The influence of host plant on gut conditions of gypsy moth (*Lymantria dispar*) caterpillars. *J. Insect Physiol*. 41:241–246.

APPEL, H. M., GOVENOR, H. L., D'ASCENZO, M. D., SISKA, E., and SCHULTZ, J. C. 2001. Limitations of folin assays of foliar phenolics in ecological studies. *J. Chem. Ecol*. 27:761–778.

AUCOIN, R. R., FIELDS, P., LEWIS, M. A., PHILOGENE, B. J. R., and ARNNASON, J. T. 1990. The protective effect of antioxidants to a phototoxin-sensitive herbivore, *Manduca sexta*. *J. Chem. Ecol*. 16:2913–2924.

AYRES, M. P. and MACLEAN, S. F., JR. 1987. Development of birch leaves and the growth energetics of *Epirrita autumnata* (Geometridae). *Ecology* 68:558–568.

BAKER, W. L. 1972. Eastern Forest Insects. USDA miscellaneous publication no. 1175. Washington, DC.

BARBEHENN, R. V. 2003. Antioxidants in grasshoppers: higher levels defend the midgut tissues of a polyphagous species than a graminivorous species. *J. Chem. Ecol*. 29:665–684.

BARBEHENN, R. V., BUMGARNER, S. L., ROOSEN, E. F., and MARTIN, M. M. 2001. Antioxidant defenses in caterpillars: Role of the ascorbate-recycling system in the midgut lumen. *J. Insect Physiol.* 47:349–357. (Erratum 47:1095)

BARBEHENN, R. V. and MARTIN, M. M. 1992. The protective role of the peritrophic membrane in the tannin-tolerant larvae of *Orgyia leucostigma* (Lepidoptera). *J. Insect Physiol*. 12:973–980.

BARBEHENN, R. V. and MARTIN, M. M. 1994. Tannin sensitivity in larvae of *Malacosoma disstria* (Lepidoptera): Roles of the peritrophic envelope and midgut oxidation. *J. Chem. Ecol.* 20:1985–2001.

BARBEHENN, R. V., MARTIN, M. M., and HAGERMAN, A. E. 1996. Reassessment of the roles of the peritrophic envelope and hydrolysis in protecting polyphagous grasshoppers from ingested hydrolyzable tannins. *J. Chem. Ecol.* 22:1901–1919.

BARBEHENN, R. V., POOPAT, U., and SPENCER, B. 2003. Semiquinone and ascorbyl radicals in the gut fluids of caterpillars measured with EPR spectrometry. *Insect Biochem. Mol. Biol*. 33:125–130.

BI, J. L. and FELTON, G. W. 1995. Foliar oxidative stress and insect herbivory: Primary compounds, secondary metabolites, and reactive oxygen species as components of induced resistance. *J. Chem. Ecol.* 21:1511–1530

CILLIERS, J. J. L. and SINGLETON, V. L. 1989. Nonenzymic autoxidative phenolic browning reactions in a caffeic acid model system. *J. Agric. Food Chem*. 37:390–396.

DADD, R. H. 1973. Insect nutrition: current developments and metabolic implications. *Annu. Rev. Entomol.* 18:381–420.

DASH, J. A. and JENNESS, R. 1985. Ascorbate content of foliage of eucalypts and conifers by some Australian and North American mammals. *Experientia* 41:952–955.

FEENY, P. P. 1968. Effect of oak leaf tannins on larval growth of the winter moth *Operophtera brumata*. *J. Insect Physiol*. 14:805–817.

FEENY, P. P. 1970. Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. *Ecology* 51:565–581.

FELTON, G. W., DONATO, K. K., DEL VECCHIO, R. J., and DUFFEY, S. S. 1989. Activation of plant polyphenol oxidases by insect feeding damage reduces the nutritive quality of foliage. *J. Chem. Ecol*. 15:2667–2694.

FELTON, G. W. and DUFFEY, S. S. 1992. Ascorbate oxidation reduction in *Helicoverpa zea* as a scavenging system against dietary oxidants. *Arch. Insect Biochem. Physiol*. 19:27–37.

FELTON, G. W. and SUMMERS, C. B. 1993. Potential role of ascorbate oxidase as a plant defense protein against insect herbivory. *J. Chem. Ecol*. 19:1553–1568.

HAGERMAN, A. E. 1988. Extraction of tannin from fresh and preserved leaves. *J. Chem. Ecol*. 14:453–461.

HAGERMAN, A. E., RIEDL, K. M., JONES, G. A., SOVIK, K. N., RITCHARD, N. T., HARTZFELD, P. W., and RIECHEL, T. L. 1998. High molecular weight plant polyphenolics (tannins) as biological antioxidants. *J. Agric. Food Chem*. 46:1887–1892.

HALLIWELL, B. and GUTTERIDGE, J. M. C. 1999. Free Radicals in Biology and Medicine. Oxford University Press, Oxford, England.

HESS, J. L. 1993. Vitamin E, α-tocopherol, pp. 111–134, *in* R. G. Alscher and J. L. Hess (eds.). Antioxidants in Higher Plants. CRC Press, Boca Raton, Florida.

HUNTER, A. F. and LECHOWICZ, M. J. 1992. Foliage quality changes during canopy development of some northern hardwood trees. *Oecologia* 89:316–323.

JOHNSON, K. S. and FELTON, G. W. 1996. Physiological and dietary influences on midgut redox conditions in generalist lepidopteran larvae. *J. Insect Physiol*. 42:191–198.

JOHNSON, K. S. and FELTON, G. W. 2001. Plant phenolics as dietary antioxidants for herbivorous insects: A test with genetically modified tobacco. *J. Chem. Ecol*. 27:2579–2597.

KAROWE, D. N. 1989. Differential effect of tannic acid on two tree-feeding Lepidoptera: Implications for theories of plant-herbivore chemistry. *Oecologia* 80:507–512.

KRAMER, K. and SEIB, P. A. 1982. Ascorbic acid and the growth and development of insects, pp. 275–291, *in* P. A. Seib and B. M. Tolbert (eds.). Ascorbic Acid: Chemistry, Metabolism and Uses. ACS, Washington, DC.

KUNERT, K. J. and EDERER, M. 1985. Leaf aging and lipid peroxidation: The role of the antioxidants vitamin C and E. *Physiol. Plant*. 65:85–88.

LINDROTH, R. L., BARMAN, M., and WEISBROD, A. W. 1991. Nutritional deficiencies in the gypsy moth, *Lymantria dispar*: Effects on larval performance and detoxication enzyme activities. *J. Insect Physiol*. 37:45–52.

LINDROTH, R. L., HSIA, M.T. S., and SCRIBER, J. M. 1987. Seasonal patterns in the phytochemistry of three *Populus* species. *Biochem. Syst. Ecol*. 15:681–686.

LINDROTH, R. L. and WEISS, A. P. 1994. Effects of ascorbic acid deficiencies on larvae of *Lymantria dispar* (Lepidoptera: Lymantriidae). *Great Lakes Entomol.* 27:169–174.

LUWE, M. 1996. Antioxidants in the apoplast and symplast of beech (*Fagus sylvatica* L.) leaves: Seasonal variations and responses to changing ozone concentrations in air. *Plant Cell Environ*. 19:321–328.

MARABOTTINI, R., SCHRAML, C., PAOLACCI, A. R., SORGONA, A., RASCHI, A., RENNENBERG, H., and BADIANI, M. 2001. Foliar antioxidant status of adult Mediterranean oak species (*Quercus ilex* L. and *Q. pubescens* Willd.) exposed to permanent CO_2-enrichment and to seasonal water stress. *Environ. Pollut.* 113:413–423.

MAUFETTE, Y. and OECHEL, W. C. 1989. Seasonal variation in leaf chemistry of the coast live oak *Quercus agrifolia* and implications for the California oak moth *Phryganidia californica*. *Oecologia* 79:439–445.

MEISTER, A. 1992. On the antioxidant effects of ascorbic acid and glutathione. *Biochem. Pharmacol.* 44:1905–1915.

NAVON, A. 1978. Effects of dietary ascorbic acid on larvae of the Egyptian cotton leafworm, *Spodoptera littoralis*. *J. Insect Physiol.* 24:39–44.

NICOL, R. W., ARNASON, J. T., HELSON, B., and ABOU-ZAID, M. M. 1997. Effect of host and non-host trees on the growth and development of the forest tent caterpillar, *Malacosoma disstria* (Lepidoptera: Lasiocampidae). *Can. Entomol*. 129:991–999.

PANZUTO, M., LORENZETTI, F., MAUFFETTE, Y., and ALBERT, P. J. 2001. Perception of aspen and sun/shade sugar maple leaf soluble extracts by larvae of *Malacosoma disstria*. *J. Chem. Ecol.* 27:1963–1978.

PARDINI, R. S. 1995. Toxicity of oxygen from naturally occurring redox-active pro-oxidants. *Arch. Insect Biochem. Physiol*. 29:101–118.

PEDERSEN, J. A. 2000. Distribution and taxonomic implications of some phenolics in the family Lamiaceae determined by ESR spectroscopy. *Biochem. Syst. Ecol.* 28:229–253.

PIETTA, P., SIMONETTI, P., and MAURI, P. 1998. Antioxidant activity of selected medicinal plants. *J. Agric. Food Chem.* 46:4487–4490.

PRATT, D. E. 1992. Natural antioxidants from plant material, pp. 54–71, *in* M.-T. Huang, C.-T. Ho, and C. Y. Lee (eds.). Phenolic Compounds in Food and their Effects on Health. II. Antioxidants and Cancer Prevention (ACS Symposium Series 507). American Chemical Soc., Washington, DC.

PRIOR, R. L., CAO G., MARTIN, A., SOFIC, E., MCEWEN, J., O'BRIEN, C., LISCHNER, N., EHLENFELDT, M., KALT, W., KREWER, G., and MAINLAND C. M. 1998. Antioxidant capacity as influenced by total phenolic and anthocyanin content, maturity, and variety of *Vaccinium* species. *J. Agric. Food Chem.* 46:2686–2693.

RE, R., PELLEGRINI, N., PROTEGGENTE, A., PANNALA, A., YANG, M., and RICE-EVANS, C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Rad. Biol. Med*. 26:1231–1237.

RICKLEFS, R. E. and MATTHEW, K. K. 1982. Chemical characteristics of the foliage of some deciduous trees in southeastern Ontario. *Can. J. Bot*. 60:2037–2045.

ROSSITER, M., SCHULTZ, J. C., and BALDWIN, I. T. 1988. Relationship among defoliation, red oak phenolics, and gypsy moth growth and reproduction. *Ecology* 69:267–277.

ROTH, S., LINDROTH, R., and MONTGOMERY, M. 1994. Effects of foliar phenolics and ascorbate on performance of the gypsy moth (*Lymantria dispar*). *Biochem. Syst. Ecol*. 22:341–351.

SAS Institute. 2000. The SAS System for Windows, Version 8e. SAS Institute, Cary, North Carolina.

SCHROEDER, L. A. 1986. Changes in tree leaf quality and growth performance of lepidopteran larvae. *Ecology* 67:1628–1636.

SCHULTZ, J. C., NOTHNAGLE, P. J., and BALDWIN, I. T. 1982. Seasonal and individual variation in leaf quality of two northern hardwood tree species. *Am. J. Bot.* 69:753–759.

SCHUPP, R. and RENNENBERG, H. 1988. Diurnal changes in the glutathione content of spruce needles (*Picea abies* L.). *Plant Sci.* 57:113–117.

SCHWANZ, P., KIMBALL, B. A., IDSO, S. B., HENDRIX, D. L., and POLLE, A. 1996a. Antioxidants in sun and shade leaves of sour orange trees (*Citrus aurantium*) after long-term acclimation to elevated CO_2. *J. Exp. Bot*. 47:1941–1950.

SCHWANZ, P., PICON, C., VIVIN, P., DREYER, E., GUEHL, J. M., and POLLE, A. 1996b. Responses of antioxidative systems to drought stress in pedunculate oak and maritime pine as modulated by elevated CO_2. *Plant Physiol.* 110:393–402.

SCRIBER, J. M. 1977. Limiting effects of low leaf-water content on the nitrogen utilization, energy budget, and larval growth of *Hyalophora cecropia* (Lepidoptera: Saturniidae). *Oecologia* 28:269–287.

SOKAL, R. R. and ROHLF, F. J. 1981. Biometry, 2 Edition. Freeman, San Francisco, California.

STEHR, F. W. and COOK, E. F. 1968. A Revision of the Genus *Malacosoma* Hübner in North America (Lepidoptera: Lasiocampidae): Systematics, Biology, Immatures, Parasites. Smithsonian Institution Press, Washington, DC.

STROHM, M., JOUANIN, L., KUNERT, K. J., PRUVOST, C., POLLE, A., FOYER, C. H., and RENNENBERG, H. 1995. Regulation of glutathione synthesis in leaves of transgenic poplar (*Populus tremula* X *P. alba*) overexpressing glutathione synthetase. *Plant J*. 7:141–145.

STRUBE, M., HAENEN, G. R. M. M., VAN DEN BERG, H., and BAST, A. 1997. Pitfalls in a method for assessment of total antioxidant capacity. *Free Rad. Res*. 26:515–521.

SUMMERS, C. B. and FELTON, G. W. 1994. Prooxidant effects of phenolic acids on the generalist herbivore *Helicoverpa zea* (Lepidoptera: Noctuiidae): Potential mode of action for phenolic compounds in plant anti-herbivore chemistry. *Insect Biochem. Mol. Biol*. 24:943–953.

SWAIN, T. 1979. Tannins and lignins, pp. 657–682, *in* G. A. Rosenthal and D. H. Janzen (eds.). Herbivores: Their Interaction with Secondary Plant Metabolites. Academic Press, New York,

THIBOLDEAUX, R. L., LINDROTH, R. L., and TRACY, J. W. 1998. Effects of juglone (5-hydroxy-1,4-naphthoquinone) on midgut morphology and glutathione status in Saturniid moth larvae. *Comp. Biochem. Physiol*. 120:481–487.

TIMMERMAN, S. E., ZANGERL, A. R., and BERENBAUM, M. R. 1999. Ascorbic and uric acid responses to xanthotoxin ingestion in a generalist and a specialist caterpillar. *Arch. Insect Biochem. Physiol*. 42:26–36.

VANDERZANT, E. S., POOL, M. C., and RICHARDSON, C. D. 1962. The role of ascorbic acid in the nutrition of three cotton insects. *J. Insect Physiol*. 8:287–297.

VELIOGLU, Y. S., MAZZA, G., GAO, L., and OOMAH, B. D. 1998. Antioxidant activity and total phenolics in selected fruits, vegetables and grain products. *J. Agric. Food Chem*. 46:4113–4117.