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ANTIOXIDANTS IN GRASSHOPPERS: HIGHER LEVELS DEFEND THE MIDGUT TISSUES OF A POLYPHAGOUS SPECIES THAN A GRAMINIVOROUS SPECIES

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Abstract—Polyphagous grasshoppers consume plants that contain markedly greater amounts of potentially prooxidant allelochemicals than the grasses eaten by graminivorous grasshoppers. Therefore, levels of antioxidant defenses maintained by these herbivores might be expected to differ in accordance with host plant ranges. Antioxidant levels were compared in midgut tissues and gut fluids of a polyphagous grasshopper, *Melanoplus sanguinipes*, and a graminivorous grasshopper, *Aulocara ellioti*. Glutathione concentrations in midgut tissues of *M. sanguinipes* (10.6 mM) are among the highest measured in animal tissues and are twice as high as those in *A. ellioti*. α-Tocopherol levels are 126% higher in midgut tissues of *M. sanguinipes* than in those of *A. ellioti*, and remain at high levels when *M. sanguinipes* is reared on plants containing a wide range of α-tocopherol concentrations. Ascorbate levels in *M. sanguinipes* midgut tissues are 27% higher than in those of *A. ellioti*, but vary depending on the host plant on which they are reared. Midgut fluids of both species contain elevated levels of glutathione, as well as large (millimolar) amounts of undetermined antioxidants that are produced in the insects. The consumption of tannic acid decreases ascorbate concentrations in midgut tisssues and gut fluids of *A. ellioti* but has no effect on ascorbate levels in *M. sanguinipes*. The results of this study provide the first measurements of antioxidants in grasshoppers and suggest that the maintenance of high levels of antioxidants in the midgut tissues of polyphagous grasshoppers might effectively protect them from oxidative stress.

Key Words—Orthoptera, *Melanoplus sanguinipes*, *Aulocara ellioti*, ascorbic acid, glutathione, α-tocopherol.

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INTRODUCTION

Research on insect defenses against ingested plant allelochemicals has focused to a large extent on detoxification and antioxidant enzymes (Lindroth, 1991; Rosenthal and Berenbaum, 1991; Felton and Summers, 1995). More recently, the importance of the acquisition and maintenance of low-molecular-weight antioxidants as defenses against potentially prooxidant plant allelochemicals has been examined. Antioxidants that have been demonstrated to protect herbivorous insects from photooxidative stress include lutein (Carroll et al., 1997), vitamin A (Green and Berenbaum, 1994), and α-tocopherol (Aucoin et al., 1995). Ascorbic acid and glutathione are believed to be important in the defense against oxidative stress from ingested phenolic compounds (Summers and Felton, 1994; Bi and Felton, 1995, 1997; Barbehenn et al., 2001).

The primary low-molecular-weight antioxidants in plants and animals are ascorbic acid (vitamin C), α-tocopherol (vitamin E), and the tripeptide glutathione. These compounds each have essential functions in the biochemical defense and nutrition of herbivorous insects (Vanderzant et al., 1962; Dadd, 1973; Kramer and Seib, 1982; Ahmad, 1992; Felton and Summers, 1995). Ascorbic acid chemically reduces a wide variety of oxidized compounds, including reactive oxygen species (ROS), α-tocopherol radical and quinone (Packer et al., 1979; Abuja and Albertini, 2001). α-Tocopherol is the major lipophilic antioxidant in biological systems and plays a key role in protecting lipids in membranes from peroxidation (Thomas et al., 1992; van Ginkel and Sevanian, 1994; Mallet et al., 1994; Glascott and Farber, 1999). Glutathione acts as a non-enzymatic radical scavenger or as a cofactor for antioxidant enzymes, such as dehydroascorbate reductase and glutathione transferase peroxidase (Meister, 1992; Ahmad, 1992; Summers and Felton, 1993; Winkler et al., 1994; Vethanayagam et al., 1999).

Phenolic compounds can act either as antioxidants or prooxidants, depending on chemical properties of the phenol (e.g., redox potential and semiquinone radical stability) and the physicochemical environment (e.g., pH, overall redox potential, oxygen tension, oxidase and peroxidase activities, and the types and concentrations of co-occurring chemical species such as catalytic metal ions, metal-chelating substances, and ascorbate). Perhaps as a result of this complexity, there are numerous reports of phenolic compounds having prooxidant effects (Hodnick et al., 1989; Canada et al., 1990; Summers and Felton, 1994; Pardini, 1995; Sakagami et al. 1997; Nose et al., 1998; Metadiewa et al., 1999) and/or antioxidant effects (Kitagawa et al., 1992; Aruoma et al., 1993; Salah et al., 1995; Guo et al., 1996; Cao et al. 1997; Hagerman et al., 1998; Aherne and O'Brien, 2000). The variety of physicochemical factors that together determine the chemical fate of ingested phenolics necessitates the measurement of one or more of the following response variables to verify prooxidant activity: (1) oxidation products (semiquinone radicals, quinones, and/or melanin like pigments), (2) ROS (peroxides, $^{\cdot}OH$, O_2^-), or

(3) biomarkers of oxidative stress. In grasshoppers, elevated levels of melanin-like pigments are produced from ingested hydrolyzable tannins (but not from gallic acid), suggesting that a substantial fraction of these tannins are oxidized (Barbehenn et al., 1996).

Useful biomarkers of oxidative stress include oxidized lipids and proteins, as well as decreased levels of antioxidants (Lauteburg et al., 1988; Lykkesfeldt et al., 1995; Glascott and Farber, 1999). A decreased level of ascorbate, for instance, may promote oxidative damage in tissues and is associated with ulcerative colitis in the human intestine (Buffinton and Doe, 1995). In insects, lower concentrations of ascorbate have been found in *Helicoverpa zea* midgut tissues in association with other markers of oxidative stress (Summers and Felton, 1994; Bi and Felton, 1995, 1997), and lower levels of ascorbate are present in the gut tissues of *Trichoplusia ni* that consumed xanthotoxin (Timmerman et al., 1999). In other insect species, such as *Heliothis virescens*, no evidence for oxidative stress has been found from ingested phenolic compounds in tobacco (Johnson and Felton, 2001). It is unclear to what extent these different results reflect differences in experimental design, e.g., phenolic compounds ingested in artificial diets or leaves, or differences in the degree of resistance to oxidative stress in different insect species.

The seminal work of Bernays and colleagues demonstrated that fatal lesions form in the midgut epithelium of graminivorous grasshoppers after long-term consumption of tannic acid (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980). Ulcerlike lesions also form in the midgut tissues of some caterpillars from the consumption of phenolic compounds (Steinly and Berenbaum, 1985; Lindroth and Peterson, 1988) and quinones (Thiboldeaux et al., 1998). The mode of action of tannic acid has been interpreted as a nonspecific association between tannins and the gut epithelium. Midgut lesions might also result from chronic oxidative stress from the oxidation of ingested tannins in the gut lumen (producing ROS) and/or the toxicity of absorbed phenols or quinones. Quinone cytotoxicity is believed to result either from sulfhydryl arylation (leading to enzyme damage and/or decreased glutathione levels) or redox cycling (producing ROS) (Gant et al., 1988; Zheng et al., 1997). Oxidative stress in either case causes elevated levels of apoptosis or necrosis and can lead to the formation of ulcerlike lesions (Babbs, 1992; Das et al., 1997; Gardner et al., 1997; Madesh et al., 1999; Chandra et al., 2000).

Previous work on antioxidants in herbivorous insects has focused primarily on larval Lepidoptera, and no examination of antioxidants in grasshoppers has been made, to my knowledge. This study tests the hypothesis that polyphagous grasshoppers, such as *Melanoplus sanguinipes* (Melanoplinae), maintain higher levels of antioxidants than do graminivorous grasshoppers, such as *Aulocara elliotti* (Gomphocerinae), and that levels of antioxidants in polyphagous species are less susceptible to decreases than are those in graminivorous species when they consume tannic acid. The basis for expecting that these insects have evolved

different levels of defense against oxidative stress lies in the distinct differences in the allelochemical profiles of their host plants. Individual polyphagous grasshoppers commonly consume a wide range of herbaceous plants (Gangwere et al., 1976; Joern 1983; Bernays and Bright, 1993). These host plants include species that produce potentially prooxidant allelochemicals, such as phenolic compounds, furanocoumarins, and terthiophenes (Lee and Berenbaum, 1990; Ahmad, 1992; Summers and Felton, 1994; Aucoin et al., 1995). By comparison, graminivorous grasshoppers feed almost exclusively on mature grasses. Because mature grasses commonly contain small amounts and limited types of allelochemicals (Bernays and Barbehenn, 1987), grass-specialist grasshoppers might derive less benefit from maintaining high levels of antioxidants compared to polyphagous grasshoppers.

METHODS AND MATERIALS

Insects

Eggs of *M. sanguinipes* were obtained from a non diapause colony (USDA, Bozeman, Montana, USA). Insects were reared on romaine lettuce (*Lactuca sativa* var. *longifolia*) and wheat bran in the fall, as previously described (Barbehenn et al., 1996). Third-instar nymphs of *A. ellioti* were collected in central Wyoming, USA, in the spring and reared to adulthood in Ann Arbor, Michigan, USA in the same conditions as *M. sanguinipes*, with the exception that the host plant was seedling wheat (*Triticum aestivum*). Wheat was grown in ambient light for experiments on *A. ellioti*, and under a mixture of ambient and supplemental light in a greenhouse for *M. sanguinipes*. Adult females were used for all experiments, unless otherwise noted. Each experimental insect was placed in a separate ventilated plastic container (470 ml) in an incubator (28°C, 16L:8D) for 2–2.5 days. Insects were assigned at random to feed on control or treated wheat leaves. Seedling wheat leaves ($N = 4$ or 5) were cut and treated with 13 μl of 70% acetone (control) or 13 μl of a 70% acetone solution of tannic acid (Sigma, lot 64F-0049) to give a final concentration of 15% dry weight (DW). Fresh control or treated leaves were placed loosely in each container daily, and these were usually completely consumed by both species.

Sample Collection

Insects that had fed recently were selected for dissection. Each insect was chilled at −20°C for 10 min, following which the entire gut was removed from the body, rinsed in distilled water (2–3 sec), blotted dry, and the Malpighian tubules removed. Samples of foregut and midgut contents were weighed in tared microcentrifuge tubes flushed with N_2. Midgut contents included the portion of the food bolus that extended into the anterior hindgut. Ascorbate and glutathione

were extracted from samples by vigorous shaking (10 sec) in 400 μl of 5% (w/v) metaphosphoric acid (MPA) and 1 mM EDTA with 50 mg/ml hydrated insoluble polyvinylpolypyrolidone (PVP). Extracts of gut contents in MPA were incubated for a minimum of 5 min on ice, with occasional mixing, to remove tannic acid. α-Tocopherol and total antioxidants in gut fluids were extracted into 325 μl of N_2-purged (1 min/ml) ethanol. Ethanol extracts were centrifuged (8000g, 2 min), and 200-μl aliquots of supernatant solutions were mixed with 10 μl of BHT (4.0 mM final concentration) for α-tocopherol measurement. Total antioxidant capacity was measured in aliquots of ethanol extracts that were not treated with BHT. Midgut tissues, including the ceca, were blotted dry on a paper towel, weighed, and homogenized in 300 μl of 5% MPA (without PVP) or 400 μl of N_2-purged ethanol in a glass hand-held tissue homogenizer (20 strokes). Aliquots (150 μl) of tissue extracts were mixed with 10 μl of BHT (5.3 mM final concentration) for α-tocopherol measurement. Wheat used as food for the insects ($N = 3$ control or tannin-treated leaves) and lettuce were weighed and homogenized in 500 μl MPA and treated with 25 mg PVP (for ascorbate and glutathione) or in 500 μl N_2-purged ethanol (50 strokes). All samples were centrifuged (8000g, 3 mins) and stored at -75°C, with ethanol extracts under N_2. Sample sizes are presented in Tables 2–5. Reagents for all assays were purchased from Sigma-Aldrich Chemical Co., Acros Organics, or ICN Pharmaceuticals.

Water

Representative samples of wheat and lettuce leaves ($N = 3$/treatment) were weighed fresh, and then reweighed after drying at 65 or 70°C. The percent water in fore- and midgut contents and midgut tissues from control insects ($N = 4$–5) of each species was measured on weighed pieces of aluminum foil before and after drying. Fluid volumes in samples were calculated (percent water $\times$ fresh weight) in order to calculate antioxidant molarity.

Antioxidants

Ascorbic Acid. Frozen MPA extracts were thawed, mixed, centrifuged (8000g, 5 min), and kept on ice. Filtered samples (0.45 μm, Gelman GHP) were mixed with 2 M Tris buffer (pH 9.2, 26% v/v) and analyzed immediately with reverse-phase HPLC (Lykkesfeldt et al., 1995; Levine et al., 1999). Ascorbate was separated with a Vydac C-18 column (201 HS, 250 $\times$ 4.6 mm) and guard column, using a mobile phase composed of aqueous ammonium phosphate (20 mM) and EDTA (1.0 mM), adjusted to pH 3.0 with 5% MPA (containing 1 mM EDTA). The flow rate was 1.0 ml/min (35°C). Peak area was measured with a Shimadzu UV–visible detector (265 nm), and peak area was integrated with a Shimadzu C-R4A integrator. The identity of the ascorbate peak was made by analysis of ascorbate standards and confirmed by treatment of standards and

samples with ascorbate oxidase (10 EU (enzyme units)/ml neutralized sample). Peak areas were converted to nanomoles injected using ascorbate standard curves. Samples were analyzed within 1–3 months from the time of storage. Previous studies have shown that ascorbate is stable after at least 6 months of storage in similar conditions (Lykkesfeldt and Ames, 1999). In this study, ascorbate remained at 87 and 98% of initial levels in tannin-treated and control wheat leaf extracts, respectively, after a period of 12 months' storage.

Glutathione. Frozen MPA extracts were thawed, mixed, and centrifuged. Aliquots (125 μl) of sample supernatant solutions were derivatized with iodoacetic acid and 2,4-dinitrofluorobenzene (Reed et al., 1980). Glutathione (GSH) and glutathione disulfide (GSSG) derivatives were analyzed with HPLC (Reed et al., 1980). Aliquots (30 μl) of filtered derivatized samples were injected onto an Alltech amino column (250 $\times$ 4.6 mm) with a C-18 guard column. Peaks were detected at 365 nm, and peak areas were converted to nanomolos injected using glutathione standard curves. Total glutathione concentrations (GSH + 2 $\times$ GSSH) reported in this study include GSSG that was present in the original samples and that which was formed during sample preparation. Samples were analyzed within 7–11 months from the time of storage. Previous studies have shown that glutathione is stable for at least 9–12 months in acidic solutions at -60° to -80°C (Brehe and Burch, 1976; Roberts and Francetic, 1993). The stability of glutathione was not tested further in this study. Glutathione levels in *A. ellioti* were measured in insects that fed on 10% DW tannic acid-treated or control wheat. An enzymatic recycling assay, adapted for use on a microplate reader, was also used to measure glutathione in the midgut tissues of *M. sanguinipes* (Griffith, 1983). Tissue samples were collected from insects as described above, with the exception that samples were homogenized in 300 μl of 5% (w/v) sulfosalicylic acid, and samples were stored (-75°C) for $<$2 weeks before analysis.

α-Tocopherol. Samples were filtered with CR PTFE filters (0.2 μm, Gelman) to HPLC vials, and the headspace flushed with N_2. Aliquots (20 or 30 μl) were injected on a Vydak C-18 column with a guard column, and eluted with 100% methanol (1.0 ml/min, 30°C), as modified from Aucoin et al. (1990). Peaks were measured with a Shimadzu fluorescence detector (294 nm excitation, 325 nm emission wavelengths), and integrated with a Shimadzu C-R4A integrator. Peak areas were converted to nanomoles of α-tocopherol injected with α-tocopherol standard curves. Samples and standards were stored for 1–5 months. α-Tocopherol was found to be stable in extracts of *L. sativa* (99.9% recovery after a 4-month storage period) and wheat (*Triticum aestivum*) (90.9% recovery after 7 month storage).

Total Antioxidant Capacity (TAC). TAC was measured with the method of Re et al. (1999). Briefly, an aqueous solution of 2,2′-azino-bis(3-ethylbenthiazoline-6-sulfonic acid (ABTS) was prepared with potassium persulfate to create the stable $ABTS^{\cdot+}$ radical cation. Absorbance (655 nm) of a stock solution of $ABTS^{\cdot+}$

was adjusted to 0.7 with ethanol. Ethanolic extracts that were stored at -75°C were remixed and centrifuged (8000g, 3 min, ambient temperature). Three replicate aliquots (6 μl) of samples or Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) standards were mixed with 190 μl of the $ABTS^{\cdot+}$ solution in 96-well microplates. After a period of 9 min in the dark (ambient temperature), absorbance was measured at 655 nm with a Biorad Benchmark microplate reader. Decolorization of the $ABTS^{\cdot+}$ solution is proportional to the concentration of "antioxidants" present in a sample, and TAC was expressed as an equivalent concentration of Trolox using a Trolox standard curve. Samples were analyzed within 1–36 days from the time of storage. TAC measurements were found to be stable in wheat samples (95.8% recovery, $N = 9$) after storage for 4 months.

Effect of Host Plant on Antioxidant Levels in M. sanguinipes

Wheat grown with supplemental light in a greenhouse for *M. sanguinipes* contained higher levels of ascorbate than wheat grown in ambient light. To test the possibility that higher ascorbate levels in the midgut tissues of *M. sanguinipes* resulted from feeding for a 2-day period on wheat containing a high concentration of ascorbate, the measurement of ascorbate was repeated in the spring using wheat grown in the same conditions as that used in experiments on *A. ellioti*. To test the long-term effect of feeding on wheat or lettuce, groups of *M. sanguinipes* from the same egg source were either reared exclusively on seedling wheat and dried seedling wheat (instead of wheat bran) or reared as usual on lettuce and wheat bran. Adult females ($N = 10$) from both rearing regimes were placed individually in containers in an incubator and fed control wheat blades for 2–2.5 days. Midgut tissues were dissected and ascorbate extracted and analyzed as described above. This experiment was repeated to compare α-tocopherol concentrations in the midgut tissues of *M. sanguinipes* reared on seedling wheat or lettuce. α-Tocopherol was extracted and analyzed as described above. Representative wheat and lettuce leaf samples ($N = 3$/species) were extracted and analyzed in each experiment.

Statistical Analyses

Analyses of antioxidant concentrations and percent water were made with SAS version 8e (SAS, 2000). Antioxidant concentrations and percentages were compared using repeated measures ANOVA, with site as the repeated measure within each insect. Species, treatment, and site were used as fixed factors in the model, which included all two-way and three-way interactions. Paired comparisons were made using the differences of least-squares means with the Proc Mixed module (SAS, 2000).

RESULTS

Water Content. Similar water content was found in wheat in each experiment and in the foregut and midgut contents and midgut tissues of *A. ellioti* and *M. sanguinipes* (Table 1). Changes in water content between the foregut and midgut contents were small and would not explain the changes in antioxidant concentrations measured along the length of the gut. The lower water content in the foregut than in the food ingested was presumably due to dehydration that occurred between the time of presentation of clipped leaves and the time they were ingested.

Ascorbic Acid. When reared on lettuce, *M. sanguinipes* maintains higher levels of ascorbate in its midgut tissues than does *A. ellioti* ($P = 0.034$, Table 2). Higher ascorbate levels in the foregut of *M. sanguinipes* presumably reflect the higher levels of ascorbate in the wheat ingested in the experiment on this species (Table 2). As a percentage of levels in food, ascorbate levels in foregut fluids of *A. ellioti* (88%) and *M. sanguinipes* (93%) were not significantly different on untreated wheat ($P = 0.530$, Table 2). Tannic acid consumption significantly decreased ascorbate concentrations in *A. ellioti* but not in *M. sanguinipes*; ascorbate levels were reduced in *A. ellioti* midgut tissues by 20.5% ($P = 0.020$), in foregut fluid by 25.0% ($P = 0.051$), and in midgut fluid by 39.1% ($P = 0.001$). In *M. sanguinipes*, ascorbate levels in the midgut fluids were only 18–30% of those in *A. ellioti* (Table 2), but remained unaffected by ingested tannic acid.

Glutathione. M. sanguinipes maintains 10.6 mM glutathione in its midgut tissues, approximately twice the level of glutathione found in *A. ellioti* ($P < 0.001$) (Table 3). Results of an enzymatic assay for glutathione in the midgut tissues of *M. sanguinipes* were similar to those measured with HPLC: 11.4 ± 0.7 mM in control tissues and 13.4 ± 0.8 mM in the tissues of insects that consumed tannins. By contrast, gut fluids of *A. ellioti* contained more glutathione than those of *M. sanguinipes*; midgut fluids in *A. ellioti* that fed on tannin-treated wheat were 2.5-fold greater than those of *M. sanguinipes* ($P < 0.001$), and there is a trend

TABLE 1. WATER CONTENTS IN WHEAT LEAVES, GUT CONTENTS, AND MIDGUT TISSUES OF *A. ellioti* AND *M. sanguinipes*[a]

Species	Site	Water (%)	*N*
Triticum aestivum	Leaf	88.2 ± 0.4	12
A. ellioti	Foregut contents	75.3 ± 3.5	4
A. ellioti	Midgut contents	81.8 ± 1.1	4
A. ellioti	Midgut tissue	75.8 ± 1.4	5
M. sanguinipes	Foregut contents	77.8 ± 1.4	5
M. sanguinipes	Midgut contents	82.6 ± 0.6	4
M. sanguinipes	Midgut tissue	75.5 ± 1.1	5

[a] Data presented as mean $\pm$ SE. Clipped leaves without a water source were fed to insects.

TABLE 2. ASCORBATE CONCENTRATIONS IN *A. ellioti* AND *M. sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID[a]

Species	Site	Tannic acid (%)	Ascorbic acid concentration (mM)	Percent of concentration in food[b]	*N*
A. ellioti	Foregut contents	0	2.93 ± 0.15^{ef}	87.5 ± 4.5^{ef}	10
A. ellioti	Foregut contents	15	2.20 ± 0.24^{d}	61.2 ± 6.7^{d}	10
A. ellioti	Midgut contents	0	1.57 ± 0.19^{c}	47.0 ± 5.8^{c}	10
A. ellioti	Midgut contents	15	0.96 ± 0.09^{b}	27.8 ± 2.4^{b}	7
A. ellioti	Midgut tissue	0	2.89 ± 0.17^{e}	86.5 ± 5.2^{ef}	11
A. ellioti	Midgut tissue	15	2.30 ± 0.11^{d}	64.1 ± 3.0^{d}	10
M. sanguinipes	Foregut contents	0	5.20 ± 0.24^{g}	93.4 ± 4.3^{f}	10
M. sanguinipes	Foregut contents	15	4.79 ± 0.35^{g}	83.1 ± 6.1^{ef}	11
M. sanguinipes	Midgut contents	0	0.28 ± 0.02^{a}	5.0 ± 0.4^{a}	9
M. sanguinipes	Midgut contents	15	0.29 ± 0.21^{a}	4.9 ± 0.4^{a}	9
M. sanguinipes	Midgut tissue	0	3.67 ± 0.34^{f}	65.8 ± 6.1^{de}	10
M. sanguinipes	Midgut tissue	15	4.15 ± 0.30^{f}	72.1 ± 5.2^{de}	11

[a] Data presented as mean ± SE. Summary statistics in each column followed by one or more of the same letters are not significantly different.

[b] Ascorbic acid concentrations in wheat ingested by *A. ellioti* and *M. sanguinipes* were 3.44 ± 0.15 mM and 5.67 ± 0.20 mM, respectively. No differences were observed in ascorbic acid levels between control and treated wheat, and these values were pooled ($N = 6$/experiment). Percent of concentration in food is based on the concentration ingested in each separate experiment.

TABLE 3. GLUTATHIONE CONCENTRATIONS IN *A. ellioti* AND *M. sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID

Species	Site	Tannic acid (%)	Glutathione concentration (mM)[a]	Percent of concentration in food[b]	*N*
A. ellioti	Foregut contents	0	0.23 ± 0.02^{a}	43.0 ± 4.4^{a}	10
A. ellioti	Foregut contents	15	0.43 ± 0.06^{b}	80.5 ± 12.1^{b}	10
A. ellioti	Midgut contents	0	0.59 ± 0.10^{cd}	110.9 ± 19.2^{c}	8
A. ellioti	Midgut contents	15	0.83 ± 0.16^{d}	156.1 ± 29.1^{c}	7
A. ellioti	Midgut tissue	0	5.94 ± 0.38^{e}	1116.9 ± 71.4^{d}	12
A. ellioti	Midgut tissue	15	5.22 ± 0.30^{e}	980.3 ± 56.5^{d}	10
M. sanguinipes	Foregut contents	0	0.20 ± 0.02^{a}	31.8 ± 2.8^{a}	10
M. sanguinipes	Foregut contents	15	0.33 ± 0.02^{a}	53.1 ± 3.5^{ab}	10
M. sanguinipes	Midgut contents	0	0.43 ± 0.05^{bc}	69.9 ± 8.6^{ab}	9
M. sanguinipes	Midgut contents	15	0.32 ± 0.05^{ab}	52.5 ± 8.1^{ab}	9
M. sanguinipes	Midgut tissue	0	10.65 ± 0.49^{f}	1718.3 ± 79.7^{e}	10
M. sanguinipes	Midgut tissue	15	10.95 ± 0.44^{f}	1766.0 ± 70.2^{e}	11

[a] Data presented as mean ± SE. Summary statistics followed by one or more of the same letters are not significantly different. Total glutathione was calculated as $[GSH] + 2 \times [GSSG]$.

[b] Glutathione concentrations in wheat ingested by *A. ellioti* and *M. sanguinipes* were 0.53 ± 0.03 and 0.62 ± 0.04 mM, respectively. No differences were observed in glutathione concentrations between control and treated wheat, and these values were pooled ($N = 6$/experiment).

TABLE 4. α-TOCOPHEROL CONCENTRATIONS IN *A. ellioti* AND *M. sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID[a]

Species	Site	Percent Tannic acid (%)	α-Tocopherol concentration (μM)	Percent of concentration in food[b]	*N*
A. ellioti	Foregut contents	0	31.6 ± 4.7^{b}	108.5 ± 18.7^{b}	14
A. ellioti	Foregut contents	15	36.6 ± 5.3^{b}	144.6 ± 23.4^{bc}	10
A. ellioti	Midgut contents	0	35.2 ± 5.6^{b}	124.0 ± 22.2^{b}	10
A. ellioti	Midgut contents	15	24.6 ± 3.1^{ab}	94.9 ± 12.0^{b}	9
A. ellioti	Midgut tissue	0	13.2 ± 2.8^{a}	48.6 ± 12.6^{a}	9
A. ellioti	Midgut tissue	15	13.3 ± 1.6^{a}	47.1 ± 5.3^{a}	10
M. sanguinipes	Foregut contents	0	31.8 ± 3.1^{b}	96.2 ± 9.2^{b}	10
M. sanguinipes	Foregut contents	15	37.8 ± 4.1^{b}	117.2 ± 13.4^{bc}	12
M. sanguinipes	Midgut contents	0	54.8 ± 7.1^{c}	165.6 ± 21.2^{c}	11
M. sanguinipes	Midgut contents	15	41.8 ± 5.1^{bc}	128.7 ± 15.0^{bc}	12
M. sanguinipes	Midgut tissue	0	30.3 ± 4.5^{b}	91.9 ± 13.8^{b}	12
M. sanguinipes	Midgut tissue	15	29.7 ± 3.1^{b}	93.2 ± 10.3^{b}	11

[a] Data presented as mean $\pm$ SE. Tannic acid was applied to wheat at a final concentration of 15% DW. Summary statistics followed by one or more of the same letters are not significantly different.

[b] α-Tocopherol concentrations in untreated wheat averaged 32.9 ± 2.3 μM, and 30.5 ± 2.7 μM in wheat treated with tannic acid (15% DW) ($N = 9$/treatment).

towards higher glutathione levels in the midgut fluids of *A. ellioti* than in *M. sanguinipes* in insects that fed on untreated wheat ($P = 0.062$). Foregut fluids of both species contained similar levels of glutathione when they fed on untreated wheat, but increased nearly 100% in *A. ellioti* that fed on tannin-treated wheat ($P = 0.031$, Table 3). Glutathione concentrations increased by over twofold along the length of the gut in each species, with the exception of *M. sanguinipes* that fed on tannin-treated wheat. *M. sanguinipes* that ingested tannin-treated wheat maintained similar levels of glutathione along the length of the gut. Glutathione in midgut fluids of *A. ellioti* and *M. sanguinipes* were affected in opposite directions by ingested tannic acid ($P = 0.002$ for species$\times$treatment interaction), with concentration increasing in the midgut fluid of *A. ellioti* (Table 3).

α-Tocopherol. α-Tocopherol levels in *M. sanguinipes* midgut tissues were 126% greater than in those of *A. ellioti* ($P = 0.016$, Table 4). α-Tocopherol levels in the foregut and midgut fluids in both species were typically as great as, or greater than, levels in the wheat ingested (Table 4). α-Tocopherol levels in *M. sanguinipes* were significantly higher in the midgut lumen than those in *A. ellioti* ($P = 0.011$), averaging 55.7% (19.6 μM) greater in control insects and 69.9% greater in tannin-consuming insects. Tannin consumption had no significant effect on α-tocopherol concentrations in gut fluids of either species (Table 4).

Total Antioxidant Capacity. TAC was virtually the same in both species (Table 5). Tannin-treatment greatly elevated the TAC of wheat and grasshopper gut fluids ($P < 0.001$; Table 5). Surprisingly, TAC in the foregut and midgut fluids of both

TABLE 5. TOTAL ANTIOXIDANT CAPACITY (TAC) IN *A. ellioti* AND *M. sanguinipes* FED WHEAT LEAVES WITH OR WITHOUT TANNIC ACID

Species	Site	acid (%)	(mM Trolox)[a]	Percent of concentration in food[b]	*N*
A. ellioti	Foregut contents	0	27.8 ± 8.1^{a}	230.0 ± 50.0^{d}	10
A. ellioti	Foregut contents	15	204.0 ± 38.5^{bc}	176.9 ± 35.5^{bcd}	7
A. ellioti	Midgut contents	0	24.6 ± 7.2^{a}	221.8 ± 51.1^{d}	10
A. ellioti	Midgut contents	15	155.6 ± 26.7^{b}	132.8 ± 27.7^{bc}	10
A. ellioti	Midgut tissue	0	14.3 ± 2.3^{a}	127.9 ± 10.5^{b}	10
A. ellioti	Midgut tissue	15	18.8 ± 2.8^{a}	16.2 ± 3.1^{a}	10
M. sanguinipes	Foregut contents	0	14.0 ± 0.5^{a}	199.3 ± 9.6^{cd}	10
M. sanguinipes	Foregut contents	15	268.0 ± 18.3^{c}	167.8 ± 10.2^{bcd}	10
M. sanguinipes	Midgut contents	0	16.0 ± 2.0^{a}	208.3 ± 17.3^{cd}	12
M. sanguinipes	Midgut contents	15	174.3 ± 21.5^{b}	111.3 ± 15.4^{b}	12
M. sanguinipes	Midgut tissue	0	10.3 ± 1.1^{a}	140.0 ± 12.7^{bc}	12
M. sanguinipes	Midgut tissue	15	23.7 ± 1.9^{a}	14.9 ± 1.0^{a}	12

[a] Data presented as mean ± SE. TAC is the extent of $ABTS^{+}$ radical reduction measured in Trolox equivalents. Summary statistics followed by one or more of the same letters are not significantly differents.

[b] TAC of wheat across all experiments was 9.2 ± 0.9 mM (control) and 141.8 ± 8.7 mM (15% DW tannic acid).

species fed untreated wheat was at least 100% higher (14–28 mM) than the TAC of the wheat ingested (7–15 mM) (Table 5).

Effect of Host Plant on Antioxidant Levels. When *M. sanguinipes* were reared on lettuce and then fed wheat containing either 3.34 or 5.67 mM ascorbate for a 2-day period, there was no significant effect on ascorbate levels in their midgut tissues, which measured 3.41 or 3.67 mM, respectively ($P = 0.600$) (Table 2, Figure 1). However, when *M. sanguinipes* were reared on wheat (containing 3.4 mM ascorbate), the ascorbate concentration in midgut tissues was decreased ($P = 0.043$) (Figure 1). Decreased levels of ascorbate were found in wheat-reared insects despite the fact that wheat contains a 4.0-fold greater ascorbate concentration than lettuce (Figure 1). Thus, when *M. sanguinipes* and *A. ellioti* are reared solely on wheat, the ascorbate levels in their midgut tissues are similar (2.70 and 2.89 mM, respectively; $P = 0.570$). α-Tocopherol also varies between the two food plants, with the concentration in *L. sativa* being 3.4-fold greater than that in seedling wheat (Figure 1). Despite the large difference in the concentrations of this antioxidant in the two host plants, *M. sanguinipes* reared on each plant maintained similar α-tocopherol concentrations in their midgut tissues (Figure 1, $P = 0.767$).

DISCUSSION

The results of this study demonstrate that a polyphagous grasshopper, *M. sanguinipes*, maintains higher levels of antioxidants in its midgut tissues than does

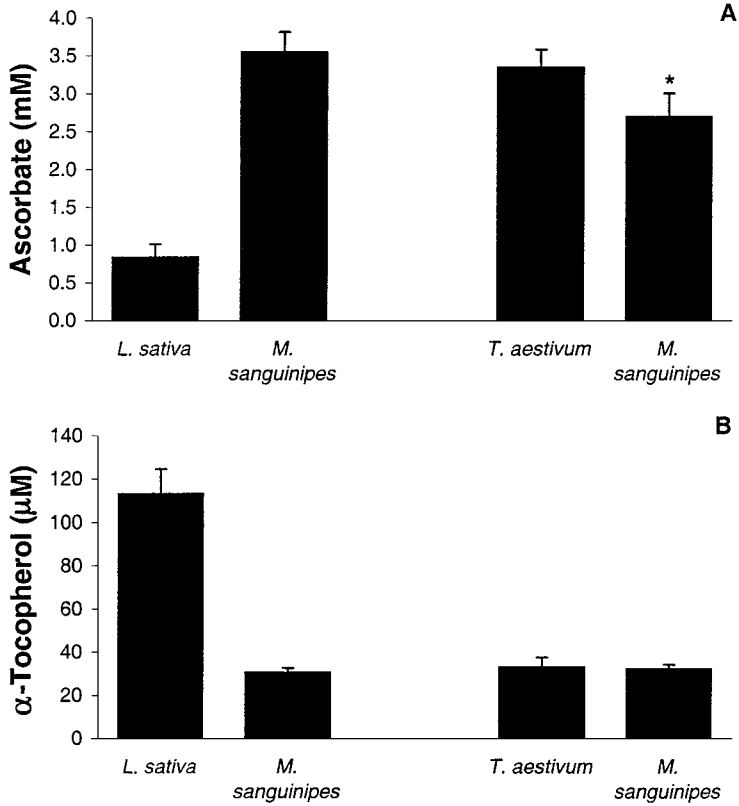

FIG. 1. (A) Ascorbate concentrations in lettuce and wheat and in *M. sanguinipes* midgut tissues from insects reared on each plant. (B) α-Tocopherol concentrations in lettuce and wheat and in *M. sanguinipes* midgut tissues from insects reared on each plant.

a graminivorous species, *A. ellioti*. Concentrations of ascorbate, α-tocopherol, and glutathione are each substantially greater in *M. sanguinipes*, consistent with the hypothesis that polyphagous grasshoppers are better defended from oxidative stress than are graminivorous species. Significant treatment effects from the consumption of tannic acid were observed in *A. ellioti*, in which ascorbate concentrations were reduced in the midgut tissues and foregut and midgut fluids. By contrast, the lack of a treatment effect on ascorbate concentrations at each of the sites measured in *M. sanguinipes* guts is consistent with the hypothesis that polyphagous species are less susceptible to oxidative stress than are graminivores. Decreased ascorbic acid in midgut tissues of tannin-consuming *A. ellioti* could result from lower levels in the gut lumen (a dietary mechanism) and/or an oxidation rate in gut tissues exceeding the insect's ability to recycle ascorbate (oxidative stress). Either

of these mechanisms could potentially lead to greater oxidative damage to the gut epithelium of *A. ellioti* than *M. sanguinipes*. Additional studies are needed to determine whether lower ascorbate levels in the midgut tissues of *A. ellioti* that consumed tannic acid for 2 days would be further aggravated in long-term feeding trials, such as those performed previously (Bernays, 1978; Bernays and Chamberlain, 1980; Bernays et al., 1980).

It is clear from this study that tannin-tolerant (polyphagous) grasshoppers in general do not depend on high levels of ascorbate in their midgut fluids as a key antioxidant defense, as has been suggested for tannin-tolerant caterpillars (Barbehenn et al., 2001). Both glutathione and ascorbate show a pattern of lower concentration in midgut fluids in *M. sanguinipes* compared to concentrations in *A. ellioti*. This result is the opposite of the pattern expected if low-molecular-weight antioxidants in the gut fluids of tannin-tolerant grasshoppers represented defenses that are necessary to permit polyphagy. Based on this study and previous results suggesting that ingested tannic acid is oxidized in *M. sanguinipes* (Barbehenn et al., 1996), it appears that well-defended midgut tissues, rather than well-defended gut fluids, are a necessary component of its antioxidant defense system. For example, higher α-tocopherol concentrations in the midgut tissues of *M. sanguinipes* might produce a greater degree of protection of the epithelial membranes from oxidative damage than is available in *A. ellioti*.

Glutathione levels in the midgut tissues of *M. sanguinipes* (10.6 mM) are among the highest recorded in animal tissues, and are nearly twice as great as those in *A. ellioti*. Intracellular glutathione in animal tissues is typically in the range of 1–8 mM (Griffith, 1999), but ranges as high as 11–12 mM in liver tissues (my calculation) (Baker et al., 1990; Roberts and Francetic, 1993). Glutathione levels in both grasshoppers examined in this study are higher than the glutathione levels in the midgut tissues of larval Lepidoptera: 0.4–1.2 mM in *Lymantria dispar* (Peric-Mataruga et al., 1997), 2.2 mM in *Manduca sexta* (Aucoin et al., 1995), and 1.2 mM in *Actias luna* and *Callosamia promethea* (Thiboldeaux et al., 1998) (my calculations). High levels of glutathione might increase the rate of product formation by a variety of antioxidant enzymes, e.g., glutathione peroxidase (if present), glutathione transferase peroxidase, and dehydroascorbate reductase, or more effectively scavenge free radicals and reduce other oxidized chemicals (Winkler et al., 1994).

Glutathione levels increased from the foregut to the midgut in three of four cases examined in this study, suggesting that this antioxidant may be secreted into the midgut lumen. Previous work on a tannin-tolerant caterpillar, *Orgyia leucostigma*, also found evidence for glutathione secretion into the gut lumen (Barbehenn et al., 2001). The trend towards elevated levels of glutathione in foreguts of tannin-consuming grasshoppers may result from regurgitation of glutathione-rich midgut fluid into the foregut (Table 3). Regurgitation into the foregut is an important process for distributing digestive enzymes, and probably

also antioxidant enzymes, in grasshoppers (Evans and Payne, 1964; Ferreira et al., 1990; Barbehenn, 2002). However, additional work is needed to determine the mechanisms underlying the patterns in antioxidant concentrations in grasshopper gut fluids. Elevated glutathione levels in tannin-fed insects, for example, could potentially result from increased damage to epithelial cells (Hagen et al., 1990). Alternatively, microbial antioxidants could be included in the extracts of gut fluids (Mead et al., 1988) in cases in which they are lysed in the extracting medium. It is also possible that elevated concentrations of antioxidants in gut fluids could result from a low efficiency of absorption of ingested antioxidants relative to other nutrients.

Levels of ascorbate vary as much as 40-fold among species of wild plants (Jones and Hughes, 1983), with species of Asteraceae (including *L. sativa*) containing an average of one third the amount of ascorbate found in species of Poaceae. Similarly, α-tocopherol varies by more than 10-fold in herbaceous plants (Mallet et al., 1994). Antioxidants vary substantially not only from one plant species to another, but also from one season to another and according to abiotic factors (e.g., Luwe, 1996; Schwanz et al., 1996a,b). The antioxidant status of polyphagous grasshoppers appears to be determined by both plant and insect factors. When *M. sanguinipes* specialize on host plants with widely varying α-tocopherol levels, they are able to maintain high levels of α-tocopherol in their midgut tissues (Figure 1), consistent with a genetically determined homeostatic mechanism. By contrast, ascorbate is neither maintained at a high level independent of its level in the host plant nor are concentrations of ascorbate in the midguts of *M. sanguinipes* directly proportional to foliar ascorbate concentrations (Figure 1). This result differs from previous work comparing *T. ni* and *Depressaria pastinacella* caterpillars, which showed that concentrations of ascorbate in gut tissues vary in proportion to levels of ascorbate in the diets of *T. ni*, and to a lesser extent in *D. pastinacella* (Timmerman et al., 1999). Previous work has shown that mixed feeding by polyphagous grasshoppers results in the acquisition of a better balance of nutrients and reduces the amounts of particular allelochemicals ingested (Bernays and Bright, 1993). It is also possible that polyphagy (mixed feeding) improves the antioxidant status of polyphagous grasshoppers in comparison to host plant specialization.

The TAC of foliage and gut fluids was greatly increased by the presence of tannic acid, a result of the strong antioxidant capacity of tannins and gallic acid in the $ABTS^{\cdot+}$ assay (Hagerman et al., 1998). A decrease in TAC from the foregut to the midgut lumen in tannin-consuming grasshoppers is consistent with the chemical transformation of ingested tannic acid (Barbehenn et al., 1996). However, the twofold increase in TAC in the foregut and midgut fluids of insects fed untreated wheat compared to the TAC of the wheat itself (Table 5) is an unexpected result and suggests that grasshoppers produce compounds in gut fluids that are potential antioxidants. The comparison between foliar and gut fluid TAC is a conservative one,

i.e., wheat leaf samples were more thoroughly extracted (macerated in ethanol) than the contents of the gut lumen (briefly extracted in ethanol by shaking). The TAC of aqueous extracts of *Heliothis virescens* midgut fluid has also been found to be greater than that of aqueous homogenates of tobacco leaves, suggesting the formation or secretion of antioxidants in caterpillar gut fluids (Johnson and Felton, 2001). However, it is less clear in this case whether concentrations of phenols and antioxidants are decreased in enzymatically active leaf extracts relative to those in gut fluids. By comparison, the $ABTS^{\cdot+}$ assay in this study used anoxic ethanolic extracts kept in an oxygen-free environment, which maintains stable TAC in plant and insect samples. In both cases, further work is needed to determine the extent to which high TAC represents high antioxidant activity *in vivo*, such as by decreasing levels of ROS or biomarkers of oxidative stress.

The doubling of the total antioxidant capacity in the gut fluids of *M. sanguinipes* and *A. ellioti* represents an increase of greater than 10,000 μM Trolox equivalents. Of the antioxidants measured in this study, only α-tocopherol was found to increase in concentration in gut fluids compared to levels in wheat leaves, but this increase is only on the order of 25 μM. Substances that are likely to be in abundance in grasshopper gut fluids that are potential antioxidants include melaninlike compounds and chlorophyll degradation products. The possibility that the black-colored "tobacco juice" of grasshoppers acts as an antioxidant is supported by extensive observations of antioxidant activity in melanins produced from oxidized phenolics such as 3,4-dihydroxyphenylalanine (DOPA). Melanins chelate metal ions, are good redox buffers, and scavenge free radicals (Sarna et al., 1976; Dunford et al., 1995; Fogarty and Tobin, 1996). The possibility that melanins act as "sacrificial antioxidants" has also been suggested previously (Dunford et al., 1995; Halliwell and Gutteridge, 1999), because oxidative damage to these molecules might occur (especially at the sites of chelated catalytic metal ions) in lieu of damage to essential nutrients and cellular components. Although high-molecular-weight melanins do not remain in ethanolic supernatant solutions (C. Felix, personal communication), the ethanolic extracts of gut fluids in this study were light brown, suggesting that low-molecular-weight melaninlike substances were retained. Pure gut fluid would contain far higher concentrations of these pigments. Chlorophyll *a* is degraded in the digestive tracts of herbivores, producing large amounts of metabolites, such as pheophytin-a (Ma and Dolphin, 1999). Further work is needed to determine whether the putative antioxidant activities of these metabolites occurs in the physicochemical conditions of the grasshopper gut lumen.

In a previous study on antioxidant enzymes in *M. sanguinipes* and *A. ellioti*, no significant differences in the activities of superoxide dismutase, catalase, ascorbate peroxidase, or glutathione transferase peroxidase were observed (Barbehenn, 2002). However, the results reported here suggest that there are additional enzymes that may enhance antioxidant levels in polyphagous grasshopper species

that should be investigated. Such enzymes might include those that maintain antioxidants in a reduced form, such as glutathione reductase, dehydroascorbate reductase, and ascorbate free radical reductase, as well as enzymes involved in glutathione synthesis, such as gluthathione synthase. The results also suggest the possibility that the costs associated with the synthesis and maintenance of large amounts of antioxidants, such as glutathione in *M. sanguinipes*, are outweighed by the low benefits to graminivorous grasshoppers because of the low levels of potentially prooxidant allelochemicals in grasses.

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