Grasshoppers efficiently process C_4 grass leaf tissues: implications for patterns of host-plant utilization

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Abstract

Leaf-chewing insects are commonly believed to be unable to crush the nutrient-rich bundle sheath cells (BSC) of C_4 grasses. This physical constraint on digestion is thought to reduce the nutritional quality of these grasses substantially. However, recent evidence suggests that BSC are digested by grasshoppers. To directly assess the ability of grasshoppers to digest C_4 grass BSC, leaf particles of *Bouteloua curtipendula* (Poaceae) were examined from the digestive tracts of two grasshopper species: *Camnula pellucida* (Scudder) (primarily a grass feeder) and *Melanoplus sanguinipes* (Fabricius) (a forb and grass generalist) (Orthoptera: Acrididae). Transmission electron microscopy was used to make the first observations of BSC crushing by herbivorous insects. *Camnula pellucida* and *M. sanguinipes* crushed over 58% and 24%, respectively, of the BSC in ingested leaf tissues. In addition, chloroplast and cell membranes were commonly disrupted in uncrushed BSC, permitting soluble nutrients to be extracted, even when BSC walls remain intact. The greater efficiency with which *C. pellucida* crushes BSC is consistent with the idea that grass-feeding species are better adapted for handling grass leaf tissues than are generalist species. By demonstrating the effectiveness with which the BSC of *B. curtipendula* can be crushed and extracted by both species of grasshoppers, this study suggests one reason why C_4 grasses are not generally avoided by grasshoppers: at least some C_4 grasses can be more easily digested than has been hypothesized.

Introduction

Grasses cover over 40% of Earth's landscapes, providing a large, but often nutritionally poor, food resource for herbivores (Williams et al., 1968; Tscharntke & Greiler, 1995). The nutritional quality of grasses is strongly affected by the biochemical and morphological characteristics associated with their photosynthetic pathways. The nutritional quality of C_4 grasses has been predicted to be poorer than that of C_3 grasses for two reasons: (1) C_4 grasses frequently have lower levels of protein, carbohydrates, and water, and higher levels of fiber and silica than C_3 grasses; and (2) the nutrients in C_4 grasses may be less digestible than those in C_3 grasses (Caswell et al., 1973; Wilson et al., 1983; Bernays & Hamai, 1987; Van Soest, 1994; Heckathorn et al., 1999; Barbehenn et al., 2004a). The lower digestibility of C_4 grasses for leaf-chewing insects is believed to result from the containment of a large fraction of the leaf's photosynthetic enzymes and other nutrients in thick-walled bundle sheath cells (BSC) (Laetsch, 1974; Ku et al., 1979; Ehleringer & Monson, 1993). Indeed, BSC have such thick walls that they can be separated from other leaf tissues by their resistance to grinding with a mortar and pestle (Berry et al., 1970).

The higher nutritional quality of C_3 grasses potentially impacts the ecology and evolution of insect herbivores as a result of direct effects on insect fitness (Slansky & Rodriguez, 1987; Joern & Gaines, 1990; Slansky, 1993; Heckathorn et al., 1999). Thus, in the large areas over which C_3 and C_4 grasses intergrade, herbivores would be expected to prefer C_3 grasses (Schoener, 1971; Caswell et al., 1973; Teeri & Stowe, 1976). However, tests of this hypothesis in the field have provided mixed results (Boutton et al., 1978; Heidorn & Joern, 1984; Pinder & Jackson, 1988), suggesting that C_4 grasses are not necessarily as poor a food resource as expected.

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The belief that the nutrients contained in the BSC in C_4 grasses are not digestible by insect herbivores (Caswell et al., 1973) is largely based on the assumption that herbivores must crush the cells in leaf tissues to digest their contents (e.g., Sibly, 1981; Hochuli, 1996). Previous work that examined the ability of grasshoppers to crush BSC concluded that their cell walls commonly remained intact and that their cell contents were largely or entirely indigestible (Caswell & Reed, 1975, 1976). However, the hypothesis that the BSC anatomy of C_4 grasses impedes the digestion of their nutrients has not been supported by studies of the efficiency of assimilation of protein and sugars by both grass specialist and generalist grasshoppers (Boys, 1981; Barbehenn et al., 2004b). Thus, the available evidence provides apparently contradictory results concerning the effect of the BSC anatomy of C_4 grasses on grasshopper nutrition.

One possible explanation for the discordant results on cell crushing and nutrient digestibility is that grasshoppers are able to extract nutrients from uncrushed BSC. Nutrients such as proteins and sugars are retained in plant cells by chloroplast and cell membranes, while cell walls are relatively porous (Carpita et al., 1979; Baron-Epel et al., 1988). Indeed, it has been established that caterpillars rapidly degrade the membranes in C_4 grass leaf tissues and efficiently extract nutrients from uncrushed BSC (Barbehenn, 1992). Thus, even if grasshoppers are unable to crush BSC, it is possible that the membranes in these cells are sufficiently disrupted during digestion to allow their nutrients to diffuse out.

Grasshoppers have been studied in many of the tests of the C_4 plant avoidance hypothesis (e.g., Caswell & Reed, 1975, 1976; Boutton et al., 1978; Heidorn & Joern, 1984; Pinder & Jackson, 1988). This study examined the leaf-processing abilities of two grasshopper species: *Camnula pellucida* (Scudder) (primarily a grass feeder) and *Melanoplus sanguinipes* (Fabricius) (a forb and grass generalist) (Orthoptera: Acrididae) (Isely, 1944; Gangwere et al., 1976; Joern, 1983; Otte, 1984). *Bouteloua curtipendula* (Michx.) Torr. (Poaceae), a PCK C_4 subtype grass, was chosen as the test food plant. These three insect and plant species are commonly found in the short-grass prairie of the western United States and southern Canada (Gould & Shaw, 1983; Otte, 1984). In addition to the ecological relevance of *B. curtipendula* to the two grasshopper species, the BSC from *B. curtipendula* appear to be digested by *M. sanguinipes* (Barbehenn et al., 2004b). To understand how some grasshoppers could digest the nutrient-rich contents of BSC from a C_4 grass, leaf particles were dissected from the digestive tracts of *M. sanguinipes* and *C. pellucida* and examined with transmission electron microscopy (TEM).

Differences between the mandible morphologies of the two grasshopper species suggested that they might have different abilities to process grass leaf tissues. *Camnula pellucida* has 'herbivorous' mandibles (with a flattened molar region and serrated incisors), while *M. sanguinipes* has 'forbivorous' mandibles (with a toothed molar region and serrated incisors) (Isely, 1944; Patterson, 1984; personal observation). The flattened molar regions of herbivorous and graminivorous mandibles are believed to help them shear apart and grind the unusually tough leaves of grasses (Patterson, 1984; Bernays & Hamai, 1987; Bernays, 1991). Since the molar region plays the major role in crushing leaf tissues in grasshoppers, it was expected that *C. pellucida* would produce more extensive physical damage to leaf tissues than would *M. sanguinipes*. Leaf tissue processing was defined both in terms of damage at the cellular level and in terms of the sizes of leaf particles produced by chewing.

Two main questions were addressed regarding the abilities of grasshoppers to access the nutrients contained in BSC: (1) Are grasshoppers able to crush the BSC from a C_4 grass? (2) Are the membranes in BSC disrupted at an early stage of digestion? In addition, a comparison of damage to leaf tissues by *C. pellucida* and *M. sanguinipes* provides a preliminary assessment of the efficiency of grass-feeding species to process grass leaf tissues vis-a-vis generalist species.

Materials and methods

Insects and grasses

Melanoplus sanguinipes were reared from eggs from a non-diapause strain (USDA, Bozeman, MT) on romaine lettuce (*Lactuca sativa*) and wheat bran (Barbehenn et al., 1996). *Camnula pellucida* were reared from eggs from field-collected insects (Lethbridge, Alberta) on wheat (*Triticum aestivum*) seedlings and bran. Adults were used in all experiments. Seed of *B. curtipendula* was obtained from the USDA-NRCS (Knox City, TX), and grasses were grown in potting soil in 20-cm-diameter pots in a greenhouse (ca. 20 plants/pot). Greenhouse temperatures ranged from an average nighttime low of 14 °C to a daytime average high of 32 °C. Grasses were placed under growth lights (L16:D8), kept well watered, and were fertilized weekly after the first month of growth. Plants used in the first experiment were approximately 2 months old (3–4 leaf stage), and in the second experiment were approximately 3 months old (4–5 leaf stage). The first and second fully expanded leaves were used for food and as control samples.

Leaf tissue damage

Grasshoppers (eight female *M. sanguinipes* and one male and female *C. pellucida*) were placed in individual plastic

containers (ca. 500 ml) and allowed to feed on wheat seedlings overnight in an incubator (28 °C, L16:D8 photoperiod). Wheat was replaced with *B. curtipendula* leaves, with their cut ends placed in tubes of water to maintain turgidity. Insects were allowed to feed for 2–5 h, sufficiently long for their midguts to be cleared of wheat leaf particles (Chapman, 1998). After chilling (10 min at −20 °C), each insect was dissected, and a sample of ingested leaf material was taken from the middle region of the foregut and midgut of each insect. Gut samples were immediately fixed in vials of 0.05 M Sorensen's phosphate-buffered glutaraldehyde (4% v/v, pH 7.2). Control leaves were cut at the time of feeding, kept in plastic tubes of water, and within an hour a central portion was cut into approximately 1 mm^2 pieces in a drop of fixative using a razor blade. Control samples were processed along with the gut samples. Fixed samples (2 h) were rinsed in phosphate buffer, stained with 1% osmium tetroxide in phosphate buffer, and dehydrated in a graded series of ethanol (30–100%). Several of the largest leaf particles from each sample were selected haphazardly for examination with TEM. Ethanol surrounding the TEM samples was replaced with propylene oxide, a mixture of propylene oxide and PolyBed 812 resin, and finally pure resin. Samples in resin were vacuum-infiltrated and placed in molds after treatment overnight in resin. Hardened embedded samples were processed further at the Biological Imaging Center (Western Michigan University, Kalamazoo, MI). Embedded samples were thin-sectioned (0.05–0.10 μm) with an LKB Ultrotome Nova Ultramicrotome. Sections were placed on 200 mesh copper grids and stained with 5% methanolic uranyl acetate and Reynolds lead citrate (Reynolds, 1963). Electron micrographs were taken with a JEOL 1230 TEM at 80 kV. The experiment was repeated after 28 days using two *M. sanguinipes* (male) and eight *C. pellucida* (three male and five female). Uncut grasses from the original planting were used as food.

A single leaf particle was observed from the foregut and midgut of each grasshopper. Within each TEM micrograph, each BSC was categorized as either having an intact wall and membranes, an intact wall but disrupted membranes, or a broken wall and disrupted membranes. Mesophyll cells were categorized as having either intact or broken cell walls, since thin sections of control samples showed that their contents were not always visible. Some BSC with broken walls may have appeared intact at the point of sectioning, making the fraction of BSC with broken walls an underestimate. Only a single leaf particle observed from a foregut in *M. sanguinipes* contained intact membranes, and this category of damage was not considered further. A total of 11 BSC and 78 mesophyll cells were observed from control samples. Fourty-eight BSC and 186 mesophyll cells were observed from the foreguts of *C. pellucida*, and 45 BSC and 124 mesophyll cells were observed from their midguts. Fifty-seven BSC and 166 mesophyll cells were observed from the foreguts of *M. sanguinipes*, and 68 BSC and 172 mesophyll cells were observed from their midguts.

Food particle and mandible size

Food particles were dissected from the foreguts of adult grasshoppers, as described previously. The foregut provided samples that were unaffected by the potential effects of 'digestive kneading' in the midgut or compaction in frass pellets. Aggregation was found to occur in the midgut contents, in which small particles no longer separated from large particles when dispersed in ethanol. In addition to the *C. pellucida* samples prepared for TEM, two *C. pellucida* (one male and one female) and 11 *M. sanguinipes* (10 male and one female) were dissected at the time of the second TEM experiment. All samples were stored at −20 °C in vials of 95% ethanol. Each sample was diluted with 95% ethanol as necessary to permit particles to lie separated when placed in small Petri dishes. Samples were first mixed to suspend the food particles, and aliquots (1 ml) of the suspended particles were transferred to a dish with a pipette. The perimeters of 100–200 particles/sample were traced on 8.5 × 11 inch paper using a Wild dissecting microscope (25 ×) with a camera lucida attachment. All particles in contact with the bottom of the dish along a linear transect were traced. The minimum particle size measured was 0.0001 mm^2, thereby including the full range of particles that were capable of settling. A small fraction of particles (estimated to be less than 10%) did not settle and were not measured. Based on a visual inspection of the particles in the ethanol overlying the settled particles (25 ×), the amounts of colloidal particles formed by the two species were not substantially different. A 1 cm square was drawn with each group of traced samples as a size standard. A total of 1404 leaf particles from *C. pellucida* and 1612 particles from *M. sanguinipes* were measured, representing total leaf surface areas of 27.86 and 24.83 mm^2, respectively. Line drawings were digitized (300 dpi) and areas were calculated with ImageJ software (http://rsb.info.nih.gov/ij/). Average particle sizes were calculated for individual grasshoppers (replicates). For clarity of presentation, particle size distributions were plotted for data pooled within each species (Figure 3). Size distributions were plotted in Microsoft Excel using a bin (subdivision) size of 0.002 mm^2, with the area of particles in each size group calculated as a fraction of the total leaf surface area ingested by each species. Exceptionally large particles (0.20–0.71 mm^2, $n = 21$ particles) from *C. pellucida* were not plotted in order to compare the lower ranges of particle sizes in the two species more clearly.

To examine whether mandible size was associated with particle size, the left mandible of each grasshopper used for measuring food particle size (n = 12 *C. pellucida* and 11 *M. sanguinipes*) was dissected. Little or no wear was apparent on these mandibles, as compared with the morphologies depicted previously (Isely, 1944; Patterson, 1984). Three measurements were made on each mandible: the distance from the posterior articulation process to the tip of the second incisor cusp (incisor length), the distance from the anterior articulation process to the tip of the first molar cusp (molar length), and the distance between the anterior and posterior articulation processes (process width) (Patterson, 1984). In addition, overall shapes of mandibles were calculated as ratios of molar length:process width and incisor length:process width (Patterson, 1984). Measurements were made with a Wild dissecting microscope with an ocular micrometer at magnifications of 6× and 12×.

Statistical analyses

In the examination of damage to cell ultrastructure, the primary comparison of interest was between species. Preliminary tests (Kruskal–Wallis) showed that there were no significant differences between experimental dates, sexes, or sites in the gut (Wilkinson, 2000). Because of the unbalanced design with respect to sex and date, and non-normal residuals that could not be transformed to meet the assumptions of ANOVA (SAS, 2000), comparisons between species were made using Kruskal–Wallis tests. The percentage of BSC and mesophyll cells with each category of damage was calculated from micrographs taken from each replicate food particle (i.e., two categories of damage to each cell type). Mean food particle size and mandible size were also compared with Kruskal–Wallis tests, using individual insects as replicates. To examine the relationship between mandible size and food particle size, principal components analysis was first used to compute a summary measure of mandible size using the five mandible measurements and size ratios for each species (PROC PRINCOMP) (SAS, 2000). Food particle size was then regressed on mandible size (principal components measure) separately for each species (SAS, 2000), using individual insects as replicates. Summary statistics are presented as the mean ± SEM.

Results

Bouteloua curtipendula has the thick-walled BSC that are typical of C_4 grasses (Figure 1A). No artifacts from preparing control samples for TEM were observed. Chloroplasts in these samples contained large numbers of starch grains, providing one indicator of the integrity of the chloroplast membranes. Both species of grasshoppers crushed the walls of BSC and mesophyll cells (Figure 1B). In BSC with intact walls, chloroplast and cell membranes were extensively disrupted in the foregut, releasing the starch grains and other nutrients from the chloroplasts. In *M. sanguinipes* only the BSC from a single food particle from the foregut contained intact chloroplast and cell membranes, and no BSC were observed in this condition in *C. pellucida*. BSC from the midguts of *C. pellucida* and *M. sanguinipes* also contained disrupted chloroplast and cell membranes (Figure 1C). Thus, there was little difference between the ultrastructure of cells in leaf tissues from the foreguts and midguts of either grasshopper species. The contents of BSC from the midgut often appeared lighter than those from the foregut, suggesting that the cytoplasmic contents were diminished. Numerous plasmodesmata perforated the cell walls of BSC, providing one route for the bulk flow and diffusion of cytoplasmic contents from these cells (Figures 1C,D).

Camnula pellucida crushed over twice as many BSC as did *M. sanguinipes* in the largest food particles ingested (0.15–0.70 mm^2) (P = 0.043) (Figure 2A). Thus, the inverse was true for the BSC that remained intact following ingestion; a larger fraction of the BSC ingested by *M. sanguinipes* remained intact and contained disrupted cell membranes than in *C. pellucida*. *Camnula pellucida* also crushed a significantly higher fraction of ingested mesophyll cells than did *M. sanguinipes* (P = 0.007) (Figure 2B). It is noteworthy that, if older leaves became tougher (MacAdam, 2002), the comparison of the two grasshopper species would provide a conservative demonstration of the greater crushing efficiency of *C. pellucida*, because eight of 10 *C. pellucida* were fed *B. curtipendula* during the second experiment.

Both grasshopper species shredded *B. curtipendula* leaf tissues into a broad range of particle sizes, the larger of which were elongate pieces centered around leaf veins (Figures 3A,B). Veins in large leaf particles from the midguts of both species were often green, suggesting that chlorophyll was retained within intact BSC. Smaller particles were generally pale colored, possibly indicating that BSC and mesophyll cells had been broken to a greater extent in these particles. Surprisingly, *M. sanguinipes* produced a larger fraction of the smallest sized particles (0.0001–0.022 mm^2) than did *C. pellucida*. In *C. pellucida* 28% of the leaf area ingested ranged from 0.0001 to 0.022 mm^2, while in *M. sanguinipes* 42% of the leaf area ingested was in this range. Thus, food particles from *M. sanguinipes* were smaller on average (0.016 ± 0.001 mm^2) than those from *C. pellucida* (0.023 ± 0.002 mm^2) (P = 0.027).

While molar lengths and mandibular process widths were greater in the *C. pellucida* than in the *M. sanguinipes* examined (P<0.05), their incisor lengths did not differ

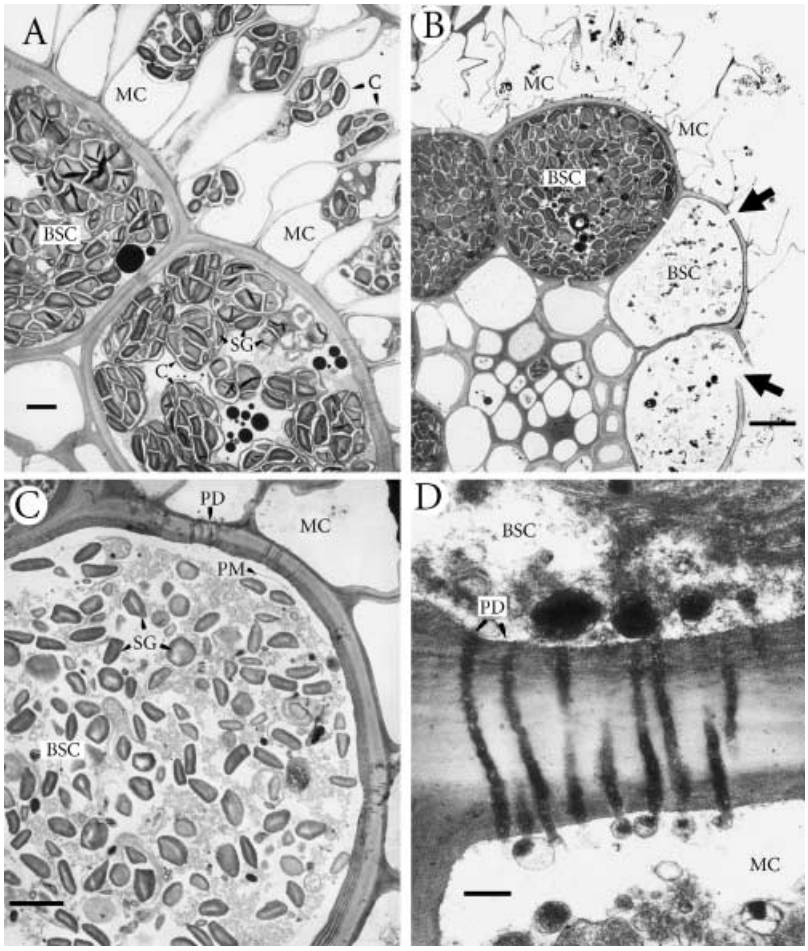

Figure 1 Representative transmission electron micrographs of the C_4 grass *Bouteloua curtipendula* before and after ingestion by *Melanoplus sanguinipes*. (A) Bundle sheath and mesophyll cells before ingestion (2500 ×, bar = 2 μm). Starch grains are constrained in groups by chloroplast membranes. (B) Bundle sheath and mesophyll cells in the foregut (1500 ×, bar = 5 μm). Mesophyll cells are broken and empty, and breaks in two BSC walls are evident (arrows). Starch grains are scattered after chloroplast membranes are disrupted. Starch digestion in the broken BSC is evident. (C) BSC from the midgut (5000 ×, bar = 2 μm). Remnants of membranes and scattered starch grains are present. (D) Plasmodesmata in the BSC wall from the midgut (40 000 ×, bar = 200 nm). The movement of BSC contents to the MC is visible. Similar observations were made in *Camnula pellucida*. BSC = bundle sheath cell, C = chloroplast, MC = mesophyll cell, PD = plasmodesmata, PM = plasma membrane, SG = starch grain.

significantly (data not shown). Although this result might be consistent with the production of smaller particles by *M. sanguinipes*, the association between mandible size and particle size did not support this conclusion. Regressions of food particle size on mandible size were not significant for either *M. sanguinipes* ($R^2 = 0.195$, $P = 0.174$) or *C. pellucida* ($R^2 = 0.046$, $P = 0.527$). The same conclusion can be drawn from a comparison of sexes within *C. pellucida*. Although mandible measurements in female *C. pellucida* averaged 19–22% greater than those in males, females and males produced leaf particle sizes that were not significantly different (0.0211 and 0.0255 mm^2, respectively). Together, these results suggest that factors other than overall mandible size and shape, such as molar morphology, determined food particle size differences between the two species.

Discussion

Despite the apparent benefits to herbivore performance from the higher nutritional quality of C_3 grasses, tests of the C_4 plant avoidance (or 'C_3–C_4') hypothesis have yielded mixed results in both field and laboratory studies (Caswell & Reed, 1975, 1976; Boutton et al., 1978; Capinera, 1978; Heidorn & Joern, 1984; Pinder & Kroh, 1987; Pinder & Jackson, 1988; Barbehenn & Bernays, 1992; Heckathorn et al., 1999; Scheirs et al., 2001; Sponheimer et al., 2003). By demonstrating the high efficiency with which the BSC of a C_4 grass can be crushed and extracted by two species of grasshoppers, this study provides one reason why C_4 grasses are not generally avoided by leaf-chewing insects: some C_4 grasses can be more easily digested than has been believed. Indeed, proteins and sugars are assimilated by *M. sanguinipes* with similar efficiencies from both *B. curtipendula* (C_4) and *Lolium multiflorum* (C_3) (Barbehenn et al., 2004b). Together with the TEM observations in this study, these results are sufficient to show that the BSC anatomy of C_4 grasses does not necessarily impede the digestion of soluble nutrients. It is noteworthy that the leaf fragments that were examined with TEM in this study were the largest and least damaged by chewing, providing a

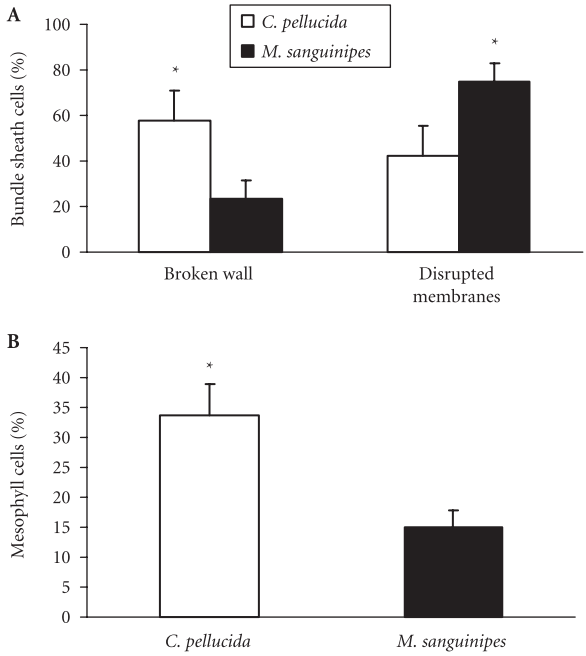

Figure 2 (A) Damage to BSC in large food particles from a C_4 grass (*Bouteloua curtipendula*) from the fore- and midguts of *Camnula pellucida* and *Melanoplus sanguinipes*. BSC either had broken cell walls and disrupted membranes, or they had intact cell walls but contained disrupted membranes. (B) Damage to mesophyll cells in the same food particles. The percentage of mesophyll cells having a broken cell wall is plotted. The remaining mesophyll cells appeared to have intact cell walls. Asterisks designate significant differences between grasshopper species ($P<0.05$).

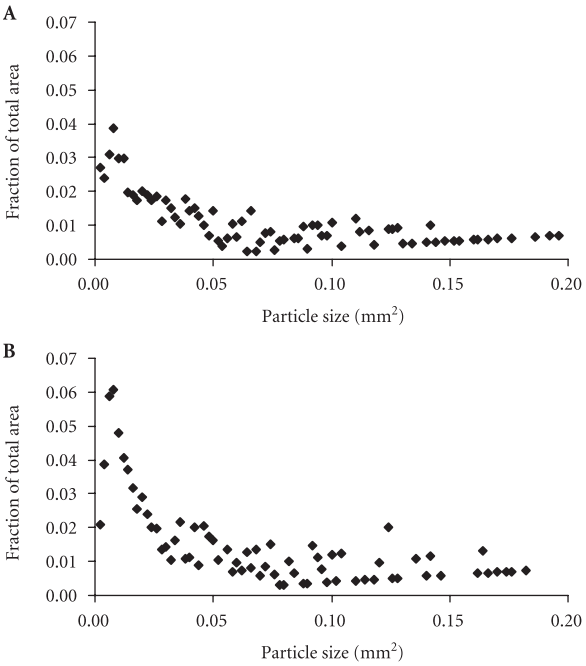

Figure 3 Food particle size distributions in (A) *Camnula pellucida* and (B) *Melanoplus sanguinipes* that consumed the C_4 grass *Bouteloua curtipendula*. The abundance of each particle size group is presented as a fraction of the sum of the areas of all particles measured in each grasshopper species.

highly conservative test of the ability of grasshoppers to crush and extract BSC.

The results of this study demonstrate that membranes in uncrushed cells are rapidly disrupted; few cells contained intact membranes in the foreguts of either grasshopper species. This finding demonstrates that grasshoppers would have time to extract nutrients from uncrushed BSC as leaf tissues pass through their digestive tracts. Plasmodesmata and pore spaces in the cell walls permit the flux of most nutrients from intact BSC and mesophyll cells once their membranes are disrupted (Robards, 1976; Carpita et al., 1979; Baron-Epel et al., 1988). The fact that *M. sanguinipes*, a generalist species that does not thoroughly crush BSC, can assimilate proteins and sugars from C_3 and C_4 grasses with similar efficiencies strongly suggests that these nutrients are efficiently extracted from uncrushed BSC. The mechanism(s) by which insects can rapidly disrupt chloroplast and cell membranes remains unknown.

Unlike proteins and sugars, starch is less efficiently digested by *M. sanguinipes* from a C_4 than a C_3 grass (Barbehenn et al., 2004b). Because starch comprises approximately two-thirds of the available (non-structural) carbohydrates in C_4 grasses (Barbehenn et al., 2004a), the retention of starch in uncrushed BSC may reduce the nutritional quality of C_4 grasses for leaf-chewing insects. Carbohydrates play important roles in insect longevity, reproduction, and dispersal (e.g., Goverde et al., 2002). Thus, if foliar carbohydrates were at limiting levels, and starch was less efficiently digested from C_4 grasses than from C_3 grasses, leaf-chewing insects might be expected to prefer C_3 grasses, all other things being equal. However, carbohydrates are known to be less important than protein for increasing demographic parameters in grasshoppers, such as egg production (Joern & Behmer, 1997). Grasshoppers are also able to compensate for widely varying food quality (Zanotto et al., 1993), although not necessarily for the full range of factors that differ between C_3 and C_4 grasses (Barbehenn et al., 2004b).

The observation that starch grains remain inside digested, but uncrushed, BSC (Figure 1C) provides an alternative explanation for previous results on BSC digestion by grasshoppers (Caswell & Reed, 1975, 1976). In these studies it was concluded that BSC are indigestible because they still

contained starch in fecal samples. However, the fraction of BSC that was crushed was not measured, the degradation of membranous structures was not visible with light microscopy, and the possibility that nutrients other than starch can diffuse from uncrushed cells was not considered previously.

An important question regards whether grasshoppers in the field would also be capable of crushing BSC and/or extracting nutrients from a variety of C_4 grasses. Environmental and C_4 grass subtype variation could affect the digestibility of C_4 grasses: (1) High growth temperature increases fiber content (Henderson & Robinson, 1982a,b), (2) NAD-ME subtype grasses are less digestible by ruminants (Wilson & Hattersley, 1983; Wilson et al., 1983), and (3) the more cubical BSC of NAD-ME grasses may be more difficult to crush than those of NADP-ME and PCK subtype grasses (Ehleringer & Monson, 1993). Neither growth temperature nor digestibility by ruminants is a factor that is likely to affect the conclusions of this study. *Bouteloua curtipendula* was grown at an average daytime high temperature of 32 °C, suggesting that temperature effects on its fiber content would have been comparable to those on grasses in their native prairie environment. More importantly, the effects of fiber, and hence C_4 grass subtype, on total dry mass digestibility in ruminants is the result of decreased cell wall digestibility (Van Soest, 1994). Leaf-chewing insects derive little, if any, nutrition from cell wall components (Martin, 1991; Clissold et al., 2004), and even large variation in grass fiber content does not affect insect nutrient utilization efficiencies (Barbehenn et al., 2004b,c). In order to generalize from the results of this study to large-scale ecological processes, further work is needed on the abilities of grasshoppers to process other C_4 grasses, including those with a range of BSC shapes. However, the finding that even one species of C_4 grass can be crushed and digested calls into question the generalization that the BSC of C_4 grasses are indigestible (Caswell et al., 1973).

Although it was reasonable to expect that the mandibles of insects would not be 'much more efficient than the biochemist's mortar and pestle' (Caswell & Reed, 1976), many, if not most, of the BSC in *B. curtipendula* were crushed by the grasshoppers in this study. The finding that the grass-feeding grasshopper *C. pellucida* is better able to crush both BSC and mesophyll cells than the generalist grasshopper *M. sanguinipes* is consistent with the idea that grass feeders are better adapted for processing C_4 grass leaf tissues than are generalist species. It would not be surprising if features such as molar morphology and mandibular muscle size contributed to chewing efficiency (Isely, 1944; Bennack, 1981; Bernays & Hamai, 1987). However, *M. sanguinipes* produces smaller food particles than *C. pellucida*, the opposite of the result expected. Based on this two-species comparison, one can only speculate that there may be trade-offs in chewing abilities between grasshoppers that feed primarily on grasses and those that feed largely on softer forbs, i.e., the flattened molar region in grass feeders may be better able to crush tough grass tissues, but the conical molar teeth of generalist feeders may be better able to triturate leaves into smaller particles. Comparative studies on additional grasshopper species, in which leaf tissue processing, consumption rate, nutrient utilization efficiency, and growth rate are measured are needed to test these ideas.

Finally, this study did not compare the abilities of early nymphal stages of grasshoppers to process C_4 grasses, and it remains possible that C_4 grasses pose a greater problem for tissue processing by young insects with relatively weak mouthparts (Bernays & Hamai, 1987). Nutrient extraction from intact BSC is potentially a more important digestive process in these insects than in adults. Clearly, different types of insects ingest leaf tissues in different ways, e.g., skeletonizing, leaf mining, snipping, and chewing (Shade & Wilson, 1967; Barbehenn, 1992; Scheirs et al., 2001), and the conclusions of this study are most relevant to late-instar leaf-chewing insects. Leaf-skeletonizing and leaf-mining insects remain clear examples of insect herbivores that are detrimentally affected by the anatomy of C_4 grasses, and which would be expected to avoid utilizing C_4 grasses (Shade & Wilson, 1967; Scheirs et al., 2001). However, in these cases the narrow spacing of the veins in C_4 grasses limits oviposition and/or feeding, and further work is needed to determine whether the digestibility of BSC plays a role in limiting the nutritional quality of C_4 grasses for insects in these feeding guilds.

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