J Chem Ecol (2016) 42:1151–1163
DOI 10.1007/s10886-016-0772-1

Spatio-Temporal, Genotypic, and Environmental Effects on Plant Soluble Protein and Digestible Carbohydrate Content: Implications for Insect Herbivores with Cotton as an Exemplar

Carrie A. Deans[1,2] · **Spencer T. Behmer**[1,3] · **Justin Fiene**[4] · **Gregory A. Sword**[1,3]

Received: 7 February 2016 / Revised: 15 May 2016 / Accepted: 7 August 2016 / Published online: 13 October 2016

Abstract Plant soluble protein and digestible carbohydrate content significantly affect insect herbivore fitness, but studies reporting plant protein and carbohydrate content are rare. Instead, the elements nitrogen and carbon often are used as surrogates for plant protein and digestible carbohydrate content, respectively. However, this is problematic for two reasons. First, carbon is found in all organic molecules, which precludes strong correlations with ecologically important dietary macronutrients (e.g., digestible carbohydrates, the primary energy source for most insect herbivores). Second, some elements (e.g., nitrogen) are present in both macronutrients (e.g., protein) and non-nutritive secondary compounds (e.g., alkaloids, protease inhibitors); in these cases N values would greatly overestimate protein available for an insect herbivore. Thus, the objective of this study was to explicitly document plant protein-carbohydrate content and assess its variation in cotton (*Gossypium hirsutum* and *G. barbadense)*, which is a nutritional resource for a number of insect herbivores. We did this by measuring plant soluble protein (P) and digestible carbohydrate (C) content across seven plant tissues, five varieties, and two growing environments. Significant differences in P and C concentration, total macronutrient content (P + C), and P:C ratio were observed across plant tissues, plant age and environment; smaller differences were seen across plant genotype. Foliar tissues had higher total P + C content compared to reproductive tissues, except for developing seeds and developing flowers, which contained twice the total P + C content; these two tissues also had the highest P content. Our data show that even agricultural monocultures offer a highly heterogeneous protein-carbohydrate landscape for insect herbivores. Characterizing plant resources using nutritional currencies (e.g., protein and carbohydrates) that are ecologically and physiologically-relevant to insect herbivores can be used to enhance our understanding of plant-insect interactions.

Keywords Cotton · *Gossypium barbadense* · *Gossypium hirsutum* · Herbivory · Nutrition · Macronutrients · Plant-insect interactions

Electronic supplementary material The online version of this article (doi:10.1007/s10886-016-0772-1) contains supplementary material, which is available to authorized users.

✉ Carrie A. Deans
cadeans@tamu.edu

[1] Department of Entomology, Texas A&M University, TAMU 2475, College Station, TX 77843, USA

[2] Department of Entomology, University of Minnesota, St. Paul, MN 55108, USA

[3] Ecology & Evolutionary Biology Graduate Program, Texas A&M University, College Station, TX 77843, USA

[4] Department of Environmental and Forest Biology, SUNY-ESF, 1 Forestry Drive, Syracuse, NY 13210, USA

Introduction

All animals eat to acquire essential nutrients. For insect herbivores, food/plant nutrient content can have a strong influence on individual performance (Behmer 2009; Clissold et al. 2009; Roeder and Behmer 2014; Simpson et al. 2004), impact community structure (Behmer and Joern 2008; Joern et al. 2012; Lenhart et al. 2015), and act as a selective force (Bernays and Chapman 1994; Jermy 1984; Warbrick-Smith et al. 2006, 2009). Insect herbivores, like all animals, require a broad suite of nutrients, but soluble protein and digestible carbohydrate (henceforth carbohydrates) are arguably two of the most important (Behmer 2009). Further evidence of their importance is that the intake of protein and carbohydrates are

tightly regulated (reviewed by Behmer 2009). Additionally, when insect herbivores eat protein and carbohydrates in optimal amounts/ratios, they show optimal reproductive output (Roeder and Behmer 2014) and greater tolerance to plant toxins (Behmer et al. 2001; Simpson et al. 2002). Finally, food protein-carbohydrate content has been implicated as a driver of mass movements (Simpson et al. 2006) and pathogen resistance (Cotter et al. 2010; Lee et al. 2006; Ponton et al. 2013; Povey et al. 2008).

Despite the ecological importance of plant soluble protein and digestible carbohydrates in shaping plant-insect interactions, few studies have explicitly measured their concentrations in plants [but see Stieger and Feller 1994 (wheat), Li et al. 1996 (legumes), Sánchez et al. 2004 (legumes), and Machado et al. 2015 (tobacco)]. Instead, elemental measures dominate, with nitrogen being used as a surrogate for protein, and carbon as a surrogate for energy. This works where there is a reasonable correlation between an element and a biomolecule (Joern et al. 2012). However, the prevalence of C in all organic molecules, and the presence of C and N in non-nutritive plant compounds, such as cellulose (which can comprise more than half the dry mass of a plant) and some allelochemicals, can make accurate conversions tenuous (Boisen et al. 1987; Ezeagu et al. 2002; Izhaki 1993; Mossé 1990). Furthermore, because insect chemoreceptors detect sugars (carbohydrates) and amino acid (from protein) concentrations, and because these macronutrients are directly tied to insect physiological function and performance, we contend that the best way to understand insect herbivore nutritional ecology is to focus on these physiologically-relevant macronutrients, rather than extrapolating from elemental data.

Additionally, the plant itself must be characterized at a scale that is relevant to the herbivore. Generally insects are smaller than the plants they consume, and they often feed selectively on specific plant tissues or structures (Bernays and Chapman 1994). In some instances, this can be rather extreme – for example, certain insect herbivores, such as early instar tobacco budworm (Hedin et al. 1991) and some weevil larvae (Hill 1987), feed primarily on reproductive structures, such as anthers or seeds For mobile insect herbivores, there also is the option of moving between plants (Bernays 1998; Bernays et al. 2004; Singer and Stireman 2001; Singer et al. 2002). For these reasons, documenting the concentration, balance, and variability of these macronutrients across several scales is essential for understanding the nutritional ecology of insect herbivores. To date, there is little information documenting variation in plant protein-carbohydrate profiles across different tissues in individual plants, not to mention differences due to environment or genotype.

Our objective in this study was to assess the protein-carbohydrate variation in cotton (*Gossypium hirsutum* and *G. barbadense*) as a resource for insect herbivores by explicitly quantifying the concentrations of soluble protein and digestible carbohydrates in different tissues, genotypes, and growing environments. The primary cotton tissues fed upon by insect herbivores are shown in Fig. 1. These include: (i) young leaves, (ii) mature leaves, (iii) squares – the developing flowers, and (iv) bolls – the fruits. Bolls themselves also contain three distinct tissues: (i) seeds, (ii) lint – fibers that aid in seed dispersal, and (iii) rind – the outer tissue of the boll. In addition to the physiological development of the entire plant, tissues near the base are older, while those nearer the top are newly developed and relatively younger. These collective tissues serve as resources for a diversity of different insect species, including chewing insects including caterpillars (e.g., *Helicoverpa armigera/H. zea*, *Spodoptera frugiperda/S. exigua*) and beetles (*Anthonomus grandis*), plus seed-feeding bugs (e.g., *Acrosternum hilare* Say, *Nezara viridula*, *Euschitus* species, and *Leptoglossus* species). As implied by the feeding classifications, different insects often specialize on specific plant structures. However, some, especially caterpillars, can feed on several different structures on a plant, which can vary depending on developmental stage (Fitt 1989). A number of hemipteran insects, including aphids (*Aphis* species), thrips (*Frankliniella* species), whiteflies (*Bemisia tabaci*), and leaf hoppers (*Pseudatomoscelis seriatus*), also feed on cotton. However, as fluid feeders they likely experience a different nutritional environment compared to insect herbivores with chewing mouthparts.

In the current study, we assessed the protein-carbohydrate variation in cotton, primarily as a resource for the main chewing insect herbivores of cotton (caterpillars and beetles) by explicitly quantifying the concentrations of soluble protein (P) and digestible-carbohydrates (C) in different cotton tissues. First, we measured the protein-carbohydrate content of different tissues across three developmental stages in two *G. hirsutum* (upland cotton) genotypes, grown under greenhouse conditions. Next, we examined protein-carbohydrate content of boll-specific tissues in the same two genotypes, including seeds, lint, and rind, at two developmental time points under greenhouse conditions. Finally, we looked at tissue protein-carbohydrate content in three genotypes of *G. hirsutum* and one genotype of *G. barbadense* (Pima cotton), all grown under field conditions. Our study fully characterizes, for the first time, soluble protein and digestible

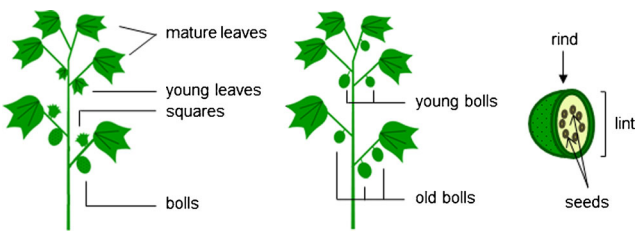

Fig. 1 Different tissues of interest in a cotton plant, all of which can be fed upon by insect herbivores. Panel (**a**) shows two leaf types (young and mature) and two reproductive structures (squares and bolls); squares are developing cotton flowers that ultimately become the fruit referred to as bolls. Panel (**b**) shows that an individual plant can simultaneously have both young and old bolls. Panel (**c**) shows the different tissues (seed, lint, and rind) that comprise a boll

carbohydrate variation within and among different plant tissues over developmental time, among different plant genotypes, and under different growing conditions. These data provide an improved context for understanding plants as nutritional resources for insect herbivores.

Methods and Materials

Experiment #1 – Protein-Carbohydrate Content in Greenhouse Grown Plants

To measure the soluble protein (P) and digestible carbohydrate (C) content of different cotton tissues, we grew two varieties of upland cotton (*Gossypium hirsutum*), a conventional variety (LA122, All-Tex Seed Co.) and a transgenic *Bt* variety (FM1740B2F, Bayer CropScience), in a greenhouse at Texas A&M University in College Station, TX, USA. It should be noted that we did not select these varieties with the expectation that the *Bt* line would be different from the conventional due to its transgenesis (the background genotype is not the same for these two varieties). They were selected simply to test for effects of genetic variation among different cultivars. Seeds were planted in potting soil (Metro-Mix 900 Professional Growing Mix) on May 11, 2012, grown to the cotyledon stage in individual planters (72 cell trays), and then transplanted to 7.5 L pots. All plants were watered with equal amounts as needed.

Eight plants from both varieties were sampled at three different time points to capture P and C content at key physiological stages: (i) 33 d after planting (DAP), before squares were present, (ii) 47 DAP, when squares were present, and (iii) 83 DAP, when bolls were present. Each plant was cut at soil-level. The node of the first fruiting branch, the number of developing flowers (i.e., squares), and number of fully developed flowers (i.e., bolls) were recorded as measures of development. All new leaves (unfurled and smaller than 6 cm in width), mature leaves (completely unfurled and wider than 6 cm), squares, and bolls (Fig. 1), were cut from each plant, placed in envelopes, and immediately frozen at −80 °C. These tissues were freeze-dried, weighed, and ground using a Wiley® Mill (Model 3383-L10, 115v, ¼ HP), before being analyzed for P and C content. The remaining stems were placed in an envelope and oven dried at 60 °C to constant mass. After all plant material was dry and weighed, the mass of the sampled tissues was combined with the mass of the stems to determine total aboveground biomass for each plant.

Experiment #2 – Protein-Carbohydrate Content of Different Boll Tissues

Insects often feed on complex plant structures comprised of different tissues, so to understand the finer scale P and C dynamics of the more complex cotton boll, we examined the macronutrient content of seed, lint, and rind across boll ages. All bolls from 15 greenhouse plants were collected at 95 DAP from both conventional (All-Tex LA122) and transgenic cotton (FM1740B3F). Bolls were categorized by their position on the plant (an indicator of tissue age) by dividing each plant into thirds. Nine bolls were selected from the top third of the plant (young bolls) and nine from the bottom third (old bolls), making sure to sample across different boll sizes. Bolls were frozen at −80 °C, freeze-dried, weighed, and dissected. The seed, lint, and rind were separated and weighed. Seed and rind were ground using a Wiley® Mill (Model 3383-L10, 115v, ¼ HP), whereas lint was cut into small pieces by hand. Each category of boll tissue was analyzed for P and C.

Experiment #3 – Protein-Carbohydrate Content in Field Grown Plants

To understand P and C dynamics in cotton grown under field conditions, we measured tissue P and C content for two different cotton species, with one of the species represented by three different varieties, or genotypes (all from All-Tex Seed Co.). On April 18th, 2012, one variety (P203) of Pima cotton (*G. barbadense*) and three varieties (LA122, LA1203, and OL220) of upland cotton (*G. hirsutum*) were planted at the Texas A&M University AgriLife Field Laboratory in Burleson, Co., TX, USA. The upland varieties included two broadleaf morphotypes (LA122 and LA1203) and one okra-leaf morphotype (OL220). Each plot contained eight 12.2 m rows separated by 1 m, with the outer two rows on each side comprising a buffer, and the inner four rows used for data collection. Seeds were planted in either monoculture plots of each variety or quad-culture plots, containing one row of each variety. Monoculture and quad-culture plots were replicated five times in a complete randomized block design across the field.

At 80 DAP, three plants of each variety from every plot were cut at soil-level, placed in garbage bags, and sealed. In each plot, one plant was collected from each end of the selected row and one from the middle of the row. Because there were different numbers of varieties between monoculture and quad-culture plots, this resulted in three plants being taken from each monoculture plot and 12 plants from the quad-culture plots (3 plants per variety). In the monoculture plots, the sampled row was selected randomly. In the quad-cultures, each row was sampled.

The plants were transported back to the laboratory and all plants were lightly washed. The number of squares, bolls, and flowers were recorded for each plant. Next, portions of the terminal growth, true leaves, squares, and bolls were dissected from each plant, pooled, placed into envelopes, and immediately put into a − 80 °C freezer. All terminal growth was harvested, but due to the large size of the mature plants, only

four true leaves, four squares, and three bolls were taken from each plant. These tissues were collected from all over the plant, but care was taken to standardize for size across fruiting structures. The collected tissue samples were freeze-dried, ground, weighed, and analyzed for P and C content in the same manner as the greenhouse samples. The rest of the plant was oven dried at 60 °C. Once all plant tissue was dried, weights were combined to determine the aboveground biomass for each variety in each plot.

Soluble Protein and Digestible Carbohydrate Analysis

Approximately 20 mg samples of ground material from each tissue type were used for the P and C assays. Soluble P content (all proteins larger than 3000 Da) was determined using the Bradford Method, as in Bradford (1976) and Compton and Jones (1985), with alterations for a plant-specific digestion protocol from Clissold et al. (2006). Concentrations of IgG (bovine gamma globulin) were used as a standard. Digestible C content (mono-, oligo-, polysaccharides, as well as methyl derivatives) was quantified using a phenol-sulfuric acid assay, as in Dubois et al. (1956) with alterations for a plant-specific digestion protocol from Clissold et al. (2006). All results are presented as percentage dry mass. The total macronutrient content, which is the combined percentages of P and C (P + C), and the P:C ratio (P/C) were calculated for each tissue for both greenhouse and field plants.

Data Analysis

Data were tested for normality. When assumptions of normality could not be met, data were either transformed or analyzed using a non-parametric Kruskall-Wallis test and/or Mann Whitney-*U* test. A Tukey HSD was used for all post hoc analyses, unless otherwise specified. All statistics were done using SPSS version 21 for Windows (SPSS Inc., Chicago, IL, USA).

For Experiment 1 (protein-carbohydrate content in greenhouse grown plants), a MANOVA was used to test for the effects of genotype, tissue, and time on protein-carbohydrate content (protein values were log-transformed to meet normality assumptions). An ANOVA was used to test the same effects on P:C ratio (log transformed) and total macronutrient content. Because all tissues were not present at all time points, separate analyses were performed for each time period.

For Experiment 2 (protein-carbohydrate content of different boll tissues), a MANOVA was used to determine the main and interactive effects of genotype and boll age on the relative dry mass of the three different boll tissues (seeds, lint, and rind). Similarly, a MANCOVA was used to determine the main and interactive effects of genotype, boll age, and tissue type on protein-carbohydrate content; boll mass was used as the covariate to account for differences in boll size. ANCOVAs were used to assess the main and interactive effects of genotype, boll age, and tissue type on P:C ratio (log transformed) and total macronutrient content; boll mass was used as the covariate.

For Experiment 3 (protein-carbohydrate content in field grown plants), a Kruskall-Wallis test was used to determine differences in aboveground dry mass across genotypes. A Bonferroni-corrected Mann Whitney-*U* test was used for post hoc comparisons. Because there was a significant effect of genotype on aboveground dry mass, plant dry mass was used as a covariate for further analysis. A MANCOVA was used to determine the main and interactive effects of genotype and tissue on protein-carbohydrate content, whereas an ANCOVA was used to assess P:C ratio (log transformed) and total macronutrient content.

Results

Experiment #1 – Protein-Carbohydrate Content in Greenhouse Grown Plants

Cotton plants were harvested for sampling at three different times. At the first harvest (33 DAP) cotton plants contained only young and mature leaves, and there was no significant genotype, tissue effect, or genotype*tissue interaction on P and C content, P:C ratio, or total macronutrient content (Table 1a). At this stage of development, both young and mature leaves were very P-rich (Fig. 2a), with P content being over three times greater than that of C content (Fig. 2b); total macronutrient (P + C) content was near 50 % for both leaf tissues (Fig. 2c).

At second harvest (47 DAP), cotton plants had young and mature leaves, plus squares, but no bolls. There was a significant effect of tissue type on P and C content, P:C ratio, and total macronutrient content, but genotype and the genotype*tissue interaction was not significant (Table 1b). The amounts of protein and carbohydrates (Fig. 2d), the P:C ratio (Fig. 2e), and total macronutrient content (Fig. 2f) were similar in young and mature leaves. Both types of leaves contained about twice as much protein as carbohydrates, and had total macronutrient content (P + C) near 50 %. Squares, by comparison, contained half as much protein as leaves (Fig. 2d), but equal amounts of protein and carbohydrates (Fig. 2e). The total macronutrient content of squares was about two-thirds that of young and mature leaves (Fig. 2i).

Finally, at the third harvest (83 DAP), cotton plants contained young and mature leaves, plus squares and bolls. A significant tissue effect was observed for P and C content and P:C ratio, but not for total macronutrient content (Table 1c); there was no significant genotype effect for these three variables, nor was the genotype*tissue interaction significant. With respect to tissue P and C content, young and

Table 1 Statistical analyses for plant protein (P) and carbohydrate (C) content, P:C ratio, and total macronutrient (P + C) content of greenhouse-grown cotton plants (*Gossypium hirsutum*)

Source	Protein & carbohydrates	P:C ratio	Total macronutrients
a) 33 Days after planting (tissues = young leaves and mature leaves)			
Genotype	$F_{2,18} = 0.56$	$F_1 = 0.68$	$F_1 = 0.37$
Tissue	$F_{2,18} = 0.98$	$F_1 = 0.59$	$F_1 = 0.42$
Genotype*Tissue	$F_{2,18} = 1.91$	$F_1 = 3.60$	$F_1 = 0.29$
b) 47 Days after planting (tissues = young leaves, mature leaves and squares)			
Genotype	$F_{2,37} = 0.31$	$F_2 = 0.70$	$F_2 = 0.57$
Tissue	$\mathbf{F_{4,76} = 5.20}$**	$\mathbf{F_2 = 10.57}$**	$\mathbf{F_2 = 6.20}$**
Genotype*Tissue	$F_{4,76} = 1.10$	$F_2 = 0.49$	$F_2 = 1.33$
a) 83 Days after planting (tissues = young leaves, mature leaves, squares and bolls)			
Genotype	$F_{2,41} = 2.04$	$F_2 = 0.48$	$F_1 = 1.53$
Tissue	$\mathbf{F_{6,84} = 8.75}$**	$\mathbf{F_2 = 29.03}$**	$F_3 = 2.28$
Genotype*Tissue	$F_{6,84} = 1.99$	$F_2 = 0.27$	$F_3 = 2.23$

[1] There were four genotypes (two varieties of upland cotton, a transgenic FM1740B2F variety, and a non-transgenic LA122 variety) for each growing period, but the number of tissues present (young leaves, mature leaves, squares and bolls) varied with plant age

[2] MANOVA results (Pillai's trace) are presented for plant protein and carbohydrate content

[3] ANOVA results are presented for plant P:C ratio and plant total macronutrient content

[4] Significant effects are shown in bold (* = $P < 0.05$; ** = $P < 0.001$)

mature leaves were similar, and both types of leaves differed compared to squares and bolls (Fig. 2g); squares and bolls also differed from one another. With respect to P:C ratio, young and old leaves were similar, and the P:C ratio of young leaves was greater compared to squares and bolls (Fig. 2h). Mature leaves had a higher P:C ratio compared to bolls, but not squares, while the P:C ratio of squares was greater than bolls (Fig. 2h).

Experiment #2 – Protein-Carbohydrate Content of Different Boll Tissues

Bolls have three different tissue types – seeds, lint (fibers attached to the seeds), and rind (Fig. 1) – so we first wanted to see if there were differences in tissue composition of bolls between genotypes (LA122 vs. FM1740B2F), and with boll age (young vs. old). A MANOVA showed a significant age effect ($F_{3,27} = 7.51$, $P = 0.001$) and a significant genotype*age interaction ($F_{3,27} = 3.39$, $P = 0.032$); genotype as a main effect was not significant ($F_{3,27} = 1.88$, $P = 0.157$). Young bolls from different genotypes varied in their tissue composition profiles (Fig. 3). Young bolls from LA122 cotton produced more rind and less seed compared to FM1740B2F cotton; lint composition was similar. Old bolls from both genotypes produced similar amount of seed, lint, and rind.

The protein-carbohydrate content of the different tissues was analyzed using a MANCOVA, which revealed a significant genotype*age*tissue interaction (Table 2). Figure 4a and b show that with respect to protein content, seeds had the highest protein content, and old seeds had 3× as much protein as young seeds (~60 and ~20 %, respectively). In contrast, lint and rind had low protein content; in fact, in all but one instance – young lint (10.8 %) – protein content for lint and rind was in the single digits. Carbohydrate content was highest in young seeds (~33 %), followed by old seeds, young lint, and young rind (ranging between 16 and 23 %); carbohydrate content was lowest in old rind (~10 %) and old lint (~3 %). Protein and carbohydrate differences as a function of genotype did exist, but these were much smaller compared to tissue type and boll age.

P:C ratio was analyzed using ANCOVA, and here we observed a significant genotype effect, and a significant age*tissue interaction (Table 2). The most notable difference was a shift in the P:C ratio of seeds as bolls aged. Seeds from young boll had a slight carbohydrate-bias, but seeds from old bolls were extremely protein-biased (Figs. 4c, d, Table S2). In contrast, the P:C ratio of rind and lint was always carbohydrate biased. The significant genotype effect, averaged across all tissues and ages, indicated that LA122 cotton had a higher P:C ratio than the FM1740B2F genotype (1.07 ± 0.04 vs. 0.94 ± 0.04).

Finally, the total macronutrient content of the seeds, lint, and rind of bolls were analyzed. We observed a significant effect of genotype, and an age*tissue interaction, with the interaction effect being particularly strong (Table 2). Total macronutrient content was always highest in the seeds (Figs. 4e, f), and seed macronutrient content increased with boll age (a 44 and 52 % increase in the LA122 and FM1740B2F genotype, respectively). In contrast, total macronutrient content in the lint and rind was ~20 % in young bolls, and dropped significantly in old bolls (Figs. 4e, f,

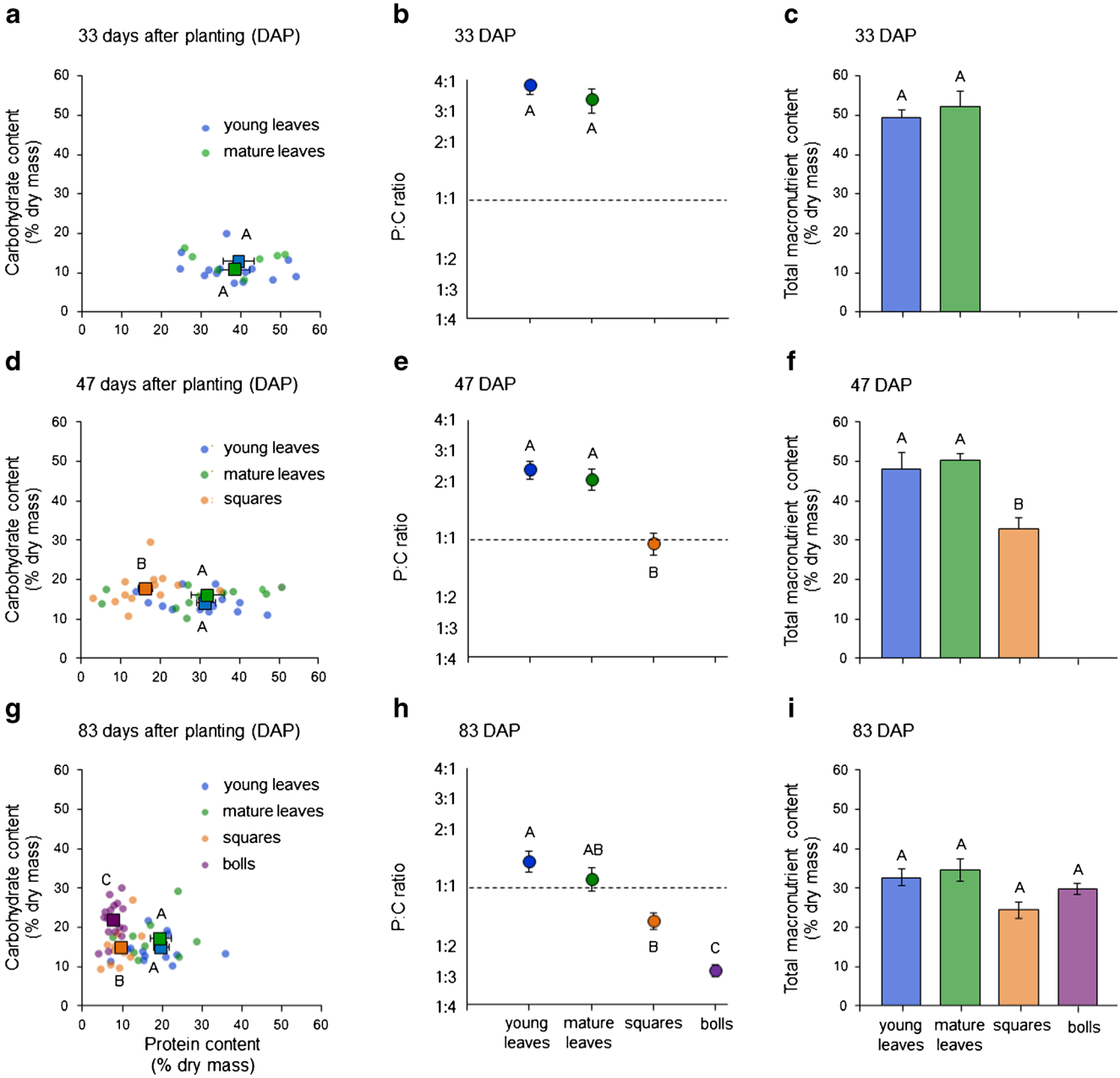

Fig. 2 Protein-carbohydrate content in greenhouse grown plants. Data are shown for three developmental time points (33 days after planting (DAP), 47 DAP, and 83 DAP). The first column of data (panels a, d, and g) are scatterplots of the protein and carbohydrate content for each tissue type (circles); the mean protein-carbohydrate content for each tissue also is shown (squares). The second column of data (panels b, e, and h) shows the mean P:C ratio for each tissue at each time point. Finally, the third column of data (panels c, f, and i) shows mean total macronutrient content (P + C) for each tissue at each time point. Variation is presented as the standard error of the mean, and different letters on each panel indicate significant differences ($P < 0.05$). Square were absent at 33 DAP, while bolls were absent at 33 and 47 DAP

Table S2); there was a particularly big drop in the macronutrient content of the lint.

Experiment #3 – Protein-Carbohydrate Content in Field Grown Plants

In this experiment, four different genotypes were grown in the field – three varieties of upland cotton (*G. hirsutum*) and one variety of Pima cotton (*G. barbadense*) – and at day 95 the young and mature leaves, squares, and bolls were harvested and analyzed for protein-carbohydrate content. Initially, we tested for differences in aboveground biomass, and found a difference (*Kruskall-Wallis test*: $H = 45.3$, $df = 3$, $P < 0.001$). Mann-Whitney *U* tests revealed that *G. barbadense* (variety P203) had the highest average dry mass (Fig. 5a). Among the three *G. hirsutum* varieties, variety LA1203 generated the

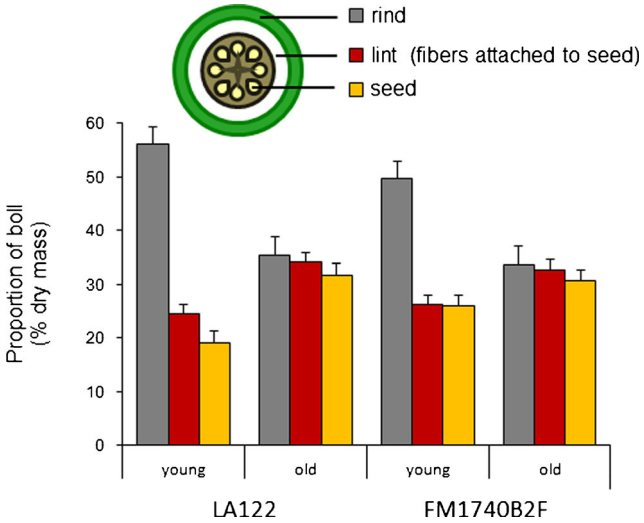

Fig. 3 Relative proportions of different boll tissues across two genotypes and two boll ages. Bolls were harvested from greenhouse grown cotton plants 95 d after planting. The figure shows the mean (±SEM) proportion of seed, lint, and rind in young and old bolls collected from two varieties of cotton, LA122 and FM1740B2F

most aboveground biomass; the remaining two varieties (OL220 and LA122) produced the least amount of aboveground biomass. Due to these significant differences, biomass was used as a covariate in the protein-carbohydrate analyses reported below.

Protein-carbohydrate content varied significantly as a function of tissue, but there was no significant genotype effect or genotype*tissue interaction (Table 3). All tissues had significantly distinct protein and carbohydrate profiles (Fig. 5b). Young leaves had the highest protein content, followed by mature leaves and squares, which were intermediate; bolls had the lowest protein content. With respect to carbohydrate, all tissues were distinct, with bolls having the highest content, followed by squares, young leaves, and mature leaves (Fig. 5b).

The P:C ratio in field plants also differed significantly among tissues, but there was no significant genotype effect, or genotype*tissue interaction (Table 3). As seen in Fig. 5c, all tissues showed distinct P:C ratios. Bolls and squares both had carbohydrate-biased ratios, while young and mature leaves had protein-biased ratios.

Finally, the total macronutrient content (P + C) differed significantly as a function of tissue type, but there was no significant genotype effect, or genotype*tissue interaction (Table 3). Total macronutrient content was highest in young leaves and bolls, slightly decreased in squares (but was significantly different compared to young leaves and bolls), and then considerably lower in mature leaves (Fig. 5d).

Discussion

For insect herbivores, soluble protein (P) and digestible carbohydrate (C) are critical nutrients. While some protein-carbohydrate data have been reported, for example, in mature leaves from plants in different watering regimes (Showler and Moran 2003) and in leaves of different growth stages in transgenic tobacco (Machado et al. 2015), collectively there still is a deficit of data available on plant protein and carbohydrate profiles, particularly at the level of detail that is ecologically relevant to insect herbivores (i.e., across different tissues, across plant development, among different varieties). In the current study, we measured the protein and carbohydrate content of cotton, a cosmopolitan agricultural crop that hosts a diverse assemblage of insect herbivores that consume a variety of plant tissues (Bottrell and Adkisson 1977; Matthews 1989). Overall, our data show that even in an agricultural

Table 2 Statistical analyses for plant protein (P) and carbohydrate (C) content, P:C ratio, and total macronutrient (P + C) content of bolls from Greenhouse-grown cotton plants (*Gossypium hirsutum*)

Source	Plant protein & carbohydrates	P:C ratio	Total macronutrients
Genotype	$\boldsymbol{F_{2,81} = 8.20}$**	$\boldsymbol{F_1 = 19.93}$**	$\boldsymbol{F_1 = 4.48}$*
Age	$\boldsymbol{F_{2,81} = 34.68}$**	$\boldsymbol{F_1 = 7.30}$**	$\boldsymbol{F_1 = 23.23}$**
Tissue	$\boldsymbol{F_{4,164} = 31.54}$**	$\boldsymbol{F_2 = 33.81}$**	$\boldsymbol{F_2 = 208.90}$**
Genotype*Age	$F_{2,81} = 1.51$	$F_1 = 1.47$	$F_1 = 0.58$
Genotype*Tissue	$F_{4,164} = 1.62$	$F_2 = 2.86$	$F_2 = 2.35$
Age*Tissue	$\boldsymbol{F_{4,164} = 15.05}$**	$\boldsymbol{F_2 = 15.39}$**	$\boldsymbol{F_2 = 49.71}$**
Genotype*Age*Tissue	$\boldsymbol{F_{4,164} = 2.58}$*	$F_2 = 2.58$	$F_2 = 2.60$
Boll mass	$F_{2,81} = 3.02$	$F_1 = 0.27$	$F_1 = 2.23$

[1] Two genotypes (a transgenic FM1740B2F variety and a non-transgenic LA122 variety), two boll ages (young and old) and three tissue types (seeds, lint and rind) were compared. All bolls were harvested 95 d after planting

[2] MANCOVA results (Pillai's trace) are presented for plant protein and carbohydrate content

[3] ANCOVA results are presented for plant P:C ratio and plant total macronutrient content. Dry boll mass was used as a covariate for all analyses

[4] Significant effects are shown in bold (* = $P < 0.05$; ** = $P < 0.001$)

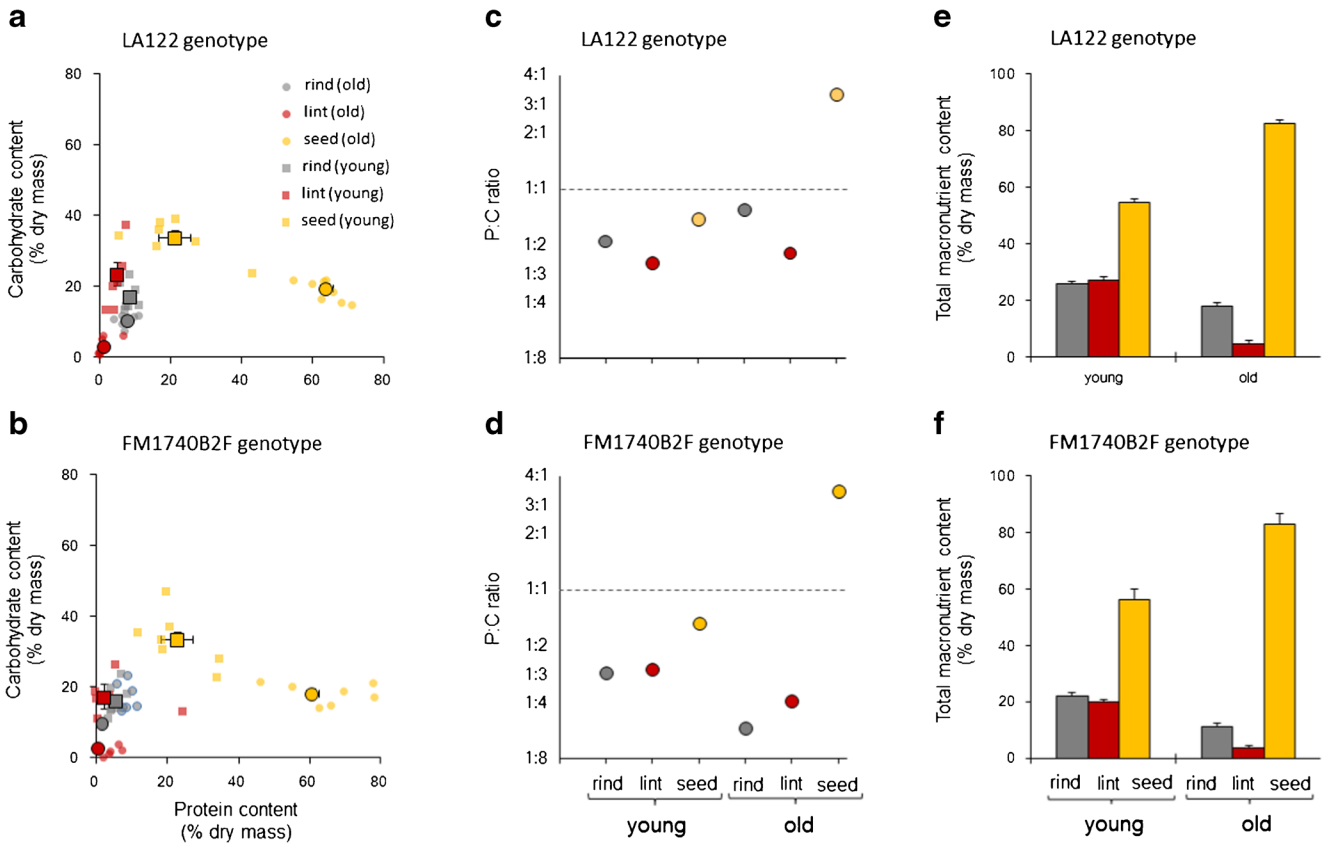

Fig. 4 Protein-carbohydrate content of different boll tissues. The average aboveground dry mass for each field genotype. Data are shown separately for two genotypes of cotton, LA122 (panels **a**, **c**, and **e**) and FM1740B2F (panels **b**, **d**, and **f**). The first row of data (panels **a** and **b**) are scatterplots of the protein and carbohydrate content for each tissue type (*seeds, lint, and rind*) from young bolls (*squares*) and old bolls (*circles*); the mean protein-carbohydrate content for each tissue also is shown (the larger squares and circles, each outlined). The second row of data (panels **c** and **d**) show the mean P:C ratio for each boll tissue type in young and old bolls (error bars are small and may be hard to detect). Finally, the third row of data (panels **e** and **f**) show the total mean total macronutrient content (P + C) for each boll tissue type in young and old bolls. Variation is presented as the standard error of the mean, and different letters on each panel indicate significant differences ($P < 0.05$)

monoculture, such as a cotton field, insects have the opportunity to forage in a highly heterogeneous nutritional landscape. We observed significant variation in protein-carbohydrate content, total macronutrient content (P + C), and P:C ratio across tissue type, plant age, genotype, and growing environment. We also found evidence that within complex tissues, such as bolls, there is strong protein-carbohydrate compartmentalization and fine-scale protein-carbohydrate dynamics. Below, we discuss how the patterns of variation in protein and carbohydrates we observed can provide insight into how insect herbivores use plants as nutritional resources, and some of the resulting ecological consequences associated with foraging in a heterogeneous protein-carbohydrate environment.

The strongest contrasts observed were between cotton tissues. In both greenhouse and field grown plants, average total macronutrient (P + C) content varied greater than 150 % between different tissues. Soluble protein content was relatively stable, varying only 1.5-fold across tissue types; however, carbohydrate content was more variable, fluctuating over 2.7-fold between bolls (highest) and mature leaves (lowest) in field-grown plants. This variability produced a 4.3-fold difference in P:C ratio between tissues, indicating that more variability exists in the proportion of protein and carbohydrates than in total P + C concentrations across tissues. Variability in P:C ratio likely has strong physiological and ecological repercussions for insect herbivores, because multiple studies have shown that diet P:C ratios have significant impact on insect performance and life history, including survival, mass gain, fecundity, and developmental time (Behmer 2009; Lee et al. 2002;; Le Gall and Behmer 2014; Roeder and Behmer 2014; Simpson and Raubenheimer 1995), as well as immunity (Cotter et al. 2010; Lee et al. 2006, 2008; Povey et al. 2008) and detoxification ability (Behmer et al. 2002; Raubenheimer 1992; Simpson and Raubenheimer 2001). Additionally, insects regulate their protein-carbohydrate intake and reach an optimal ratio (Behmer 2009; Raubenheimer and Simpson 1997; Simpson and Raubenheimer 1993). This suggests that fluctuations in plant tissue P:C ratio may have strong impact on insect feeding behavior, as individuals may need to feed on multiple plant tissues that vary in P:C ratio in order to reach their protein-carbohydrate intake target. For instance, caterpillars and plant bugs that colonize cotton in mid-season, when all plant tissues are present, tend to feed on different tissue types

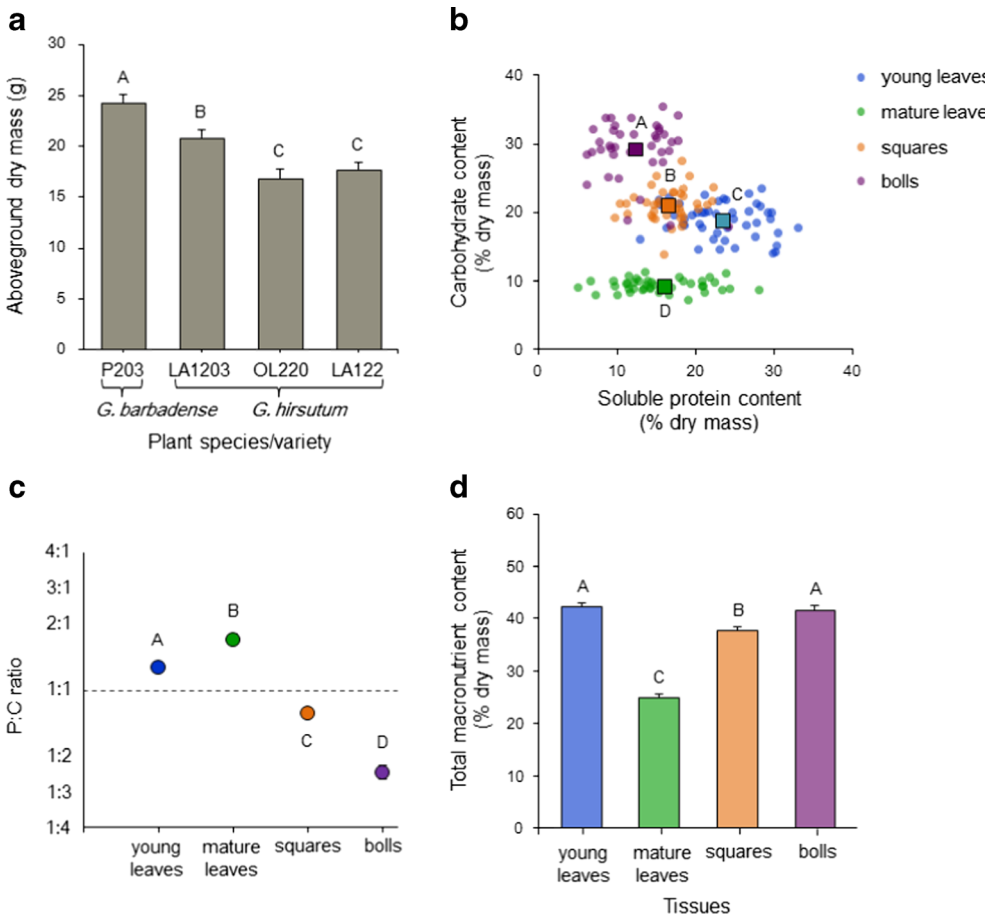

Fig. 5 Above ground plant biomass and protein-carbohydrate content in field grown cotton plants. Four genotypes were compared – three varieties of upland cotton (*Gossypium hirsutum*) and one variety of *G. barbadense*). Panel (**a**) shows the mean aboveground biomass for each species/variety. Panel (**b**) is a scatterplot of the protein and carbohydrate content for each tissue type (*circles*); the mean protein-carbohydrate content for each tissue is also shown (squares with outlines). Panel (**c**) shows the mean P:C ratio for each tissue (error bars are small and may be hard to detect). Panel (**d**) shows mean total macronutrient content (P + C) for each tissue. Variation is presented as the standard error of the mean, and different letters on each panel indicate significant differences ($P < 0.05$). There were not differences between genotypes, and the genotype*tissue interaction was not significant, so data for each tissue type are pooled across the four genotypes

throughout their development. Adult *H. zea* moths typically lay eggs on cotton squares, but newly hatched larvae tend to feed initially on terminal growth, but later switch to squares and seeds of developing bolls (Boyd et al. 2004; Quaintance and Brues 1905). Plant bugs, such as *Lygus* species, feed on both squares and developing bolls (Backus et al. 2007; Layton 2000; Snodgrass 1998). Having access to different tissue types undoubtedly provides opportunities for these species to better regulate protein and carbohydrate intake to reach an optimal ratio.

We also saw significant variability in tissue protein-carbohydrate content over time, with protein generally decreasing and carbohydrate content increasing or staying the same in the all tissues throughout the growing season. This resulted in all tissues becoming more carbohydrate-biased throughout plant development. There also was a general decrease in total macronutrient (P + C) concentration across all tissues over time. These changes likely are due to increases in nutrient allocation, particularly the differential allocation of amino acids and proteins towards fruits in older plants.

Table 3 Statistical analyses for plant protein (P) and carbohydrate (C) content, P:C ratio, and total macronutrient (P + C) content of field-grown cotton plants (*Gossypium hirsutum*)

Source	Plant protein & carbohydrates	P:C ratio	Total plant macronutrients
Genotype	$F_{6,304} = 1.493$	$F_3 = 1.374$	$F_3 = 2.165$
Tissue	$\mathbf{F_{6,304} = 86.245}$**	$\mathbf{F_3 = 142.905}$**	$\mathbf{F_3 = 78.353}$**
Genotype*Tissue	$F_{18,304} = 1.164$	$F_{10} = 1.410$	$F_{10} = 0.978$
Dry mass	$F_{2,151} = 0.843$	$F_1 = 0.206$	$F_1 = 0.036$

[1] Four genotypes were compared – three varieties of upland cotton (*Gossypium hirsutum*), and one variety of *G. barbadense*. All plants were harvested 80 d after planting, and for each plant four different tissues were collected (young leaves, mature leaves, squares and bolls)

[2] MANOVA results (Pillai's trace) are presented for plant protein and carbohydrate content

[3] ANOVA results are presented for plant P:C ratio and plant total macronutrient content

[4] Significant effects are shown in bold (* = $P < 0.05$; ** = $P < 0.001$)

These changes over the growing season suggest that there is a strong connection between plant phenology and tissue protein-carbohydrate content (Hwang et al. 1983), as temporal patterns in plant protein-carbohydrate content likely impact the temporal movement of different insect herbivore species among tissue types within a cotton plant, but also among host plant species. For early-season colonizers, such as cutworms (Lepidoptera) and thrips (Thysanaptera), young cotton leaves represents a highly nutritious resource. That mature cotton plants, starting at the base, display a vertical gradient of old to young tissues also provides an added source of variation, beyond the differences that already exist among tissue types, for mid-season species like bollworms and plant bugs. Finally, seeds were the only tissue to increase in both total macronutrient concentration and % protein, as well as overall biomass, over time. As such, older bolls provide an excellent resource for seed-feeders, such as stink bugs and leaf-footed bugs, which increase in density late in the season. Later instar *Heliothis* species, which also have a P-biased intake target similar to bollworms, feed selectively on older bolls (Wilson and Waite 1982).

In addition to the spatio-temporal protein-carbohydrate patterns observed across tissues, we also found that scale is important for accurately describing resource quality. Although we found that whole intact bolls had the lowest P:C ratio, when we took a closer look at the tissues within the boll we found compartmentalization of protein and carbohydrates. It was initially surprising that bolls appeared to be a low quality resource (carbohydrate-biased), as fruiting structures generally are considered high protein resources (Bernays and Chapman 1994); we ultimately found that the seed contained within the bolls were the most nutrient-rich of all tissues measured. In addition, the pattern of temporal variation in boll tissues was markedly different from those seen in other tissues. Whereas other tissues showed a decrease in total macronutrient content (P + C) and P:C ratio over time, this pattern was observed only for the lint and rind tissues in bolls. Seed not only increased in dry mass throughout boll development, but total macronutrient content and P:C ratio also increased with boll age, making seed the only tissue to become more nutritious throughout plant development. It is noted that the only significant genotype effect on protein-carbohydrate content we observed throughout this entire study occurred in bolls. Boll age had a stronger effect on bolls from the transgenic FM1740B2F genotype vs. the LA122 variety, particularly in regard to tissue soluble protein content (Table S1), which dropped more precipitously in the rind and lint tissues as FM1740B2F bolls aged. However, given that these varieties do not represent the transgenic and its isoline, we cannot determine whether this difference was due to transgenic events or other genetic differences between the two varieties sampled. Nevertheless, these results show that future studies must focus on specific tissues that are relevant to the scale of the insect in order to accurately characterize the protein-carbohydrate content of the resources that insect herbivores are actually ingesting. They also indicate that genotypic variation among plants may be a more important factor underlying variation in the nutritional environment for insects that feed mainly on bolls vs. foliage.

We also observed a strong effect of growing environment on plant protein-carbohydrate content, which reflects the dynamic nature of plant nutrient allocation patterns. Figure 6 shows that overall carbohydrate content was more stable in the field than in the greenhouse, while protein content varied more drastically in the field. Furthermore, some tissues showed more variability than others. For instance, the P:C ratio of mature leaves was 120 % higher in the greenhouse than the field at 80 DAP. Additionally, while the total macronutrient (P + C) content of young leaves, squares, and bolls was 18.3, 36.2, and 27.4 % lower in the greenhouse than the field, respectfully, mature leaves showed an increase in total macronutrient content of 24.2 % under greenhouse conditions. These discrepancies in protein-carbohydrate content between greenhouse and field plants indicate that differences in growing environment can strongly impact plant physiology, especially in ways that are not always apparent by observation alone. This conclusion has far-reaching implications for insect studies that utilize greenhouse plants to test field-relevant interactions, as plant protein-carbohydrate profiles in greenhouse plants may be significantly different from those in field plants, and thus, may generate confounding results. The cause for these fluctuations is not yet clear. However, as we did not observe high levels of herbivory on our fields plants, differences in abiotic factors (Preece and Sutter 1991), such as light

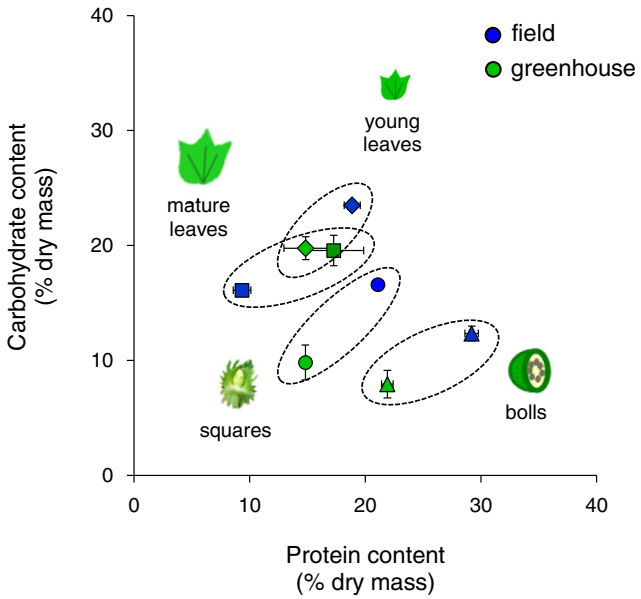

Fig. 6 Protein-carbohydrate content for each tissue from greenhouse-grown [83 days after planting (DAP)] and field-grown (80 DAP) cotton plants. Data are shown as means (±SEM)

intensity (Davies 1977), wind (Hunt and Jaffe 1980), water and nutrient availability (Bunce 1977), as well as biotic interactions with soil microbes (Bardgett and Chan 1999; Biswas et al. 2000; Egamberdiyeva 2007; Egamberdiyeva and Höflich 2004), are good candidates. Ultimately, future research in identifying the connections between these abiotic factors and protein-carbohydrate fluxes will improve our understanding of the nutritional relationship between plants and insects.

The data described in this study provide a detailed description of variation in the protein-carbohydrate environment experienced by insect herbivores; however, the values we report are really most relevant to chewing insects. Sucking insects, especially hemipterans that specialize on phloem (e.g., aphids) or xylem (e.g., spittlebugs), feed exclusively within the plant vascular system. Because we did not explicitly measure the concentration of sugars and free amino acids within plant phloem or xylem, we cannot comment on the nutritional environment available to insects feeding from these vascular structures (although see do Amarante et al. 2006; Grassi et al. 2002; Karley et al. 2002; Ponder et al. 2000; Wilkinson and Douglas 2003). Despite this, our data are relevant to those sucking insect species that practice extra-oral digestion, such as cell-rupture feeders (thrips), lacerate-and-flush feeders (*Lygus* species), and seed-feeders (various stink bug species), as they are accessing the nutrients found within plant cells (Auclair 1969). In any case, more research and better techniques are needed to fully quantify the protein-carbohydrate content of all plant resources at biologically relevant scales in order to characterize the protein-carbohydrate landscape for all types of insect herbivores.

Our study is the first to simultaneously measure plant soluble protein and digestible carbohydrate content on different spatial and temporal scales (across different plant tissues over plant developmental time), at the genotypic level (across varieties), and at the environmental level (greenhouse and field environments). The results indicate that plant protein-carbohydrate content can potentially account for a large portion of variability in the nutritional environment encountered by insect herbivores. Spatial variability in plant protein-carbohydrate content undoubtedly impacts insect performance and movement by constraining access to dietary protein and carbohydrates, and driving foraging behavior. Through these effects, plant protein-carbohydrate patterns also likely influence ecological interactions. In addition, the extensive temporal variability documented in this study suggests that plant protein-carbohydrate content plays an important role in the evolution of insect life history traits, host plant associations, and niche partitioning (Behmer and Joern 2008). An understanding of the constraints plant protein-carbohydrate content places on insect herbivores will enhance our understanding of the proximate and ultimate factors influencing insect nutritional ecology.

Finally, laboratory studies, particularly those employing the geometric framework for nutrition (Simpson and Raubenheimer 2012), have been useful for delineating insect nutritional requirements and preferences. However, there has been less progress in characterizing the actual nutritional landscapes in which insects forage. Data on variability in both concentrations and ratios of protein and carbohydrates, rather than simple elemental measures, in natural or agricultural plant communities, are lacking. The results of our study show substantial variation in key macronutrients for insect herbivores, even in a seemingly homogeneous environment like a cotton monoculture. Recognizing and incorporating this variation into experimental designs and predictive models can have implications for better understanding plant-insect interactions, ranging from insect physiology and behavioral ecology to agroecology and pest management. For example, a self-regulated P:C intake target ratio of 1.6:1 recently has been determined as the optimal diet for the caterpillar *H. zea*, a major agricultural pest of cotton and other crops in North America (Deans et al. 2015). Linking the nutritional requirements for this species to the macronutrient content of its host plants is essential for making connections between insect physiology, feeding, and performance in the field. The value of this link was illustrated recently by Deans et al. (2016) who showed that the use of ecologically-realistic artificial diets with macronutrient levels that correspond to both insect nutritional needs and availability in the field dramatically affects *H. zea* susceptibility to the Cry1Ac toxin used for its management in *Bt* transgenic crops. Results such as this highlight how knowing the protein and carbohydrate content of different host plants can be used to test hypotheses about nutritionally-mediated ecological effects, including variation in susceptibility to plant defenses, *Bt* transgenics, pathogens, and parasitoids (to name a few).

Acknowledgments We thank all who have contributed to this project, either through assistance with field collection, chemical analyses, or general feedback, including: Paul Lenhart, Marion Le Gall, Rebecca Clark, Fiona Clissold, Mickey Eubanks, Cesar Valencia, Lauren Kalns, Diana Castillo-Lopez, Maria Julissa Ek-Ramos, Nicole Locke, and Steve Hague. Charlie Cook from All-Tex Seed Inc. provided seed for use in these experiments. Aspects of this study were supported by the Biotechnology Risk Assessment Grants (BRAG) program from the U.S. Department of Agriculture (2015-33522-24099) awarded to GAS and STB, as well as the C. Everette Salyer Fellowship in Cotton Entomology and the Dissertation Fellowship offered by Texas A&M University and awarded to CAD.

References

Auclair JL (1969) Nutrition of plant-sucking insects on chemically defined diets. Entomol Exp Appl 12:623–641

Backus EA, Cline AR, Ellerseick MR, Serrano MS (2007) *Lygus hesperus* (Hemiptera: Miridae) feeding on cotton: new methods

and parameters for analysis of nonsequential electrical penetration graph data. Ann Entomol Soc Am 100:296–310
Bardgett RD, Chan KF (1999) Experimental evidence that soil fauna enhance nutrient mineralization and plant nutrient uptake in montane grassland ecosystems. Soil Biol Biochem 31:1007–1014
Behmer ST (2009) Insect herbivore nutrient regulation. Annu Rev Entomol 54:167–187
Behmer ST, Joern A (2008) Coexisting generalist herbivores occupy unique nutritional feeding niches. Proc Natl Acad Sci U S A 105: 1977–1982
Behmer ST, Raubenheimer D, Simpson SJ (2001) Frequency-dependent food selection in locusts: a geometric analysis of the role of nutrient balancing. Anim Behav 61:995–1005
Behmer ST, Simpson SJ, Raubenheimer D (2002) Herbivore foraging in chemically heterogeneous environments: nutrients and secondary metabolites. Ecology 83:2489–2501
Bernays EA (1998) Evolution of feeding behavior in insect herbivores. Bioscience 48:35–44
Bernays EA, Chapman RF (1994) Host-plant selection by phytophagous insects. Chapman & Hall, NY
Bernays EA, Singer MS, Rodrigues D (2004) Foraging in nature: foraging efficiency and attentiveness in caterpillars with different diet breadths. Ecol Entomol 29:389–397
Biswas JC, Ladha JK, Dazzo FB (2000) *Rhizobia* inoculation improves nutrient uptake and growth of lowland rice. Soil Sci Soc Am J 64: 1644–1650
Boisen S, Bech-Andersen S, Eggum BO (1987) A critical view of the conversion factor 6.25 from total nitrogen to protein. Acta Agric Scand 37:299–304
Bottrell DG, Adkisson PL (1977) Cotton insect pest management. Annu Rev Entomol 22:451–481
Boyd ML, Phipps BJ, Wrather JA, Newman M, Sciumbato GL (2004) Cotton pests: Scouting and management. Extension publications, University of Missouri
Bradford MM (1976) A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254
Bunce JA (1977) Leaf elongation in relation to leaf water potential in soybean. J Exp Bot 28:156–161
Clissold FJ, Sanson GD, Read J (2006) The paradoxical effects of nutrient ratios and supply rates on an outbreaking insect herbivore, the Australian plague locust. J Anim Ecol 75:1000–1013
Clissold FJ, Sanson GD, Read J, Simpson SJ (2009) Gross vs. net income: how plant toughness affects performance of an insect herbivore. Ecology 90:3393–3405
Compton SJ, Jones CG (1985) Mechanism of dye response and interference in the Bradford protein assay. Anal Biochem 151:369–374
Cotter SC, Simpson SJ, Raubenheimer D, Wilson K (2010) Macronutrient balance mediates trade-offs between immune function and life history traits. Funct Ecol 25:186–198
do Amarante L, Lima JD, Sodek L (2006) Growth and stress conditions cause similar changes in xylem amino acids for different legume species. Env. Exp Biol 58:123–129
Davies WJ (1977) Stomatal responses to water stress and light in plants grown in controlled environments and in the field. Crop Sci 17:735–740
Deans CA, Sword GA, Behmer ST (2015) Revisiting macronutrient regulation in the polyphagous herbivore *Helicoverpa zea* (Lepidoptera: Noctuidae): new insights via a nutritional geometry. J Insect Physiol 81:21–27
Deans CA, Sword GA, Behmer ST (2016) Nutrition as a neglected factor in insect herbivore susceptibility to *Bt* toxins. Curr Opin Insect Sci 15:97–103
Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F (1956) Colormetric method for determination of sugars and related substances. Anal Chem 28:350–358
Egamberdiyeva D (2007) The effect of plant growth promoting bacteria on growth and nutrient uptake of maize in two different soils. Appl Soil Ecol 36:184–189
Egamberdiyeva D, Höflich G (2004) Effect of plant growth-promoting bacteria on growth and uptake of cotton and pea in a semi-arid region of Uzbekistan. J Arid Environ 56:293–301
Ezeagu IE, Petzke JK, Metges CC, Akinsoyinu AO, Ologhobo AD (2002) Seed protein contents and nitrogen-to-protein conversion factors for some uncultizated tropical plant seeds. Food Chem 78: 105–109
Fitt GP (1989) The ecology of *Heliothis Species* in relation to agroecosystems. Annu Rev Entomol 34(1):17–53
Grassi G, Millard P, Wendler R, Minotta G, Tagliavini M (2002) Measurement of xylem sap amino acid concentrations in conjunction with whole tree transpiration estimates spring N remobilization by cherry (*Prunus avium* L.) trees. Plant Cell Environ 25:1689–1699
Hedin PA, Parrott WL, Jenkins JN (1991) Effects of cotton plant allelochemicals and nutrients on behavior and developments of tobacco budworm. J Chem Ecol 17:1171–1121
Hill DS (1987) Agricultural insect pests of temperate regions and their control. Cambridge University Press, UK
Hunt ER, Jaffe MJ (1980) Thigmomorphogenesis: The interaction of wind and temperature in the field on the growth of *Phaseolus vulgaris* L. Ann Bot-London 45:665–672
Hwang BK, Ibenthal WD, Heitefuss R (1983) Age, rate of growth, carbohydrate and amino acid contents of spring barley plants in relation to their resistance to powdery mildew (*Erysiphe graminis* f.Sp. hordei. Physiol Plant Pathol 22:1–14
Izhaki I (1993) Influence of nonprotein nitrogen on estimation of protein from total nitrogen in fleshy fruits. J Chem Ecol 19:2605–2615
Jermy T (1984) Evolution of insect/host plant relationships. Am Nat 124
Joern A, Provin T, Behmer ST (2012) Not just the usual suspects: insect herbivore populations and communities are associated with multiple plant nutrients. Ecology 93:1002–1015
Karley AJ, Douglas AE, Parker WE (2002) Amino acid composition and nutritional quality of potato leaf phloem sap for aphids. J Exp Biol 205:3009–3018
Layton MB (2000) Biology and damage of the tarnished plant bug, *Lygus lineolaris*. cotton Southwest Entomol 23:7–20
Le Gall M, Behmer ST (2014) Effects of protein and carbohydrate on an insect herbivore: the vista from a fitness landscape. Integr Comp Biol 54:942–954
Lee KP, Behmer ST, Simpson SJ, Raubenheimer D (2002) A geometric analysis of nutrient regulation in the generalist caterpillar *Spodoptera littoralis* (Boisduval. J Insect Physiol 48:655–665
Lee KP, Cory JS, Wilson K, Raubenheimer D, Simpson SJ (2006) Flexible diet choice offsets protein costs of pathogen resistance in a caterpillar. Proc Roy Soc Lond B Bio 273:823–829
Lee KP, Simpson SJ, Wilson K (2008) Dietary protein-quality influences melanization and immune function in an insect. Funct Ecol 22: 1052–1061
Lenhart PA, Eubanks MD, Behmer ST (2015) Water stress in grasslands: dynamic responses of plants and insect herbivores. Oikos 124:381–390
Li R, Volenec JJ, Joern BC, Cunningham SM (1996) Seasonal changes in nonstructural carbohydrates, protein, and macronutrients in roots of alfalfa, red clover, sweetclover, and birdsfoot trefoil. Crop Sci 36: 617–623
Machado AR, Arce CCM, Ferrieri AP, Baldwin IT, Erb M (2015) Jasmonate-dependent depletion of soluble sugars compromises plant resistance to *Manduca sexta*. New Phytol 207:91–105
Matthews GA (1989) Cotton insect pests and their management. Longman Scientific and Technical, UK
Mossé J (1990) Nitrogen to protein conversion factor for ten cereals and six legume or oilseeds. A reappraisal of its definition and

determination. Variation according to species and to seed protein content. J Agric Food Chem 38:18–24

Ponder KL, Pritchard J, Harrington R, Bale JS (2000) Difficulties in location and acceptance of phloem sap combined with reduced concentration of phloem amino acids explain lowered performance of the aphid *Rhopalosiphum padi* on nitrogen deficient barely (*Hordeum vulgare*) seedlins. Entomol Exp Appl 97:203–210

Ponton F, Wilson K, Holmes AJ, Cotter SC, Raubenheimer D, Simpson SJ (2013) Integrating nutrition and immunology: a new frontier. J Insect Physiol 59:130–137

Povey S, Cotter SC, Simpson SJ, Lee KP, Wilson K (2008) Can the protein costs of bacterial resistance be offset by altered feeding behavior? J Anim Ecol 78:437–446

Preece JE, Sutter EG (1991) Acclimatization of micropropagated plants to the greenhouse and field. In: Debergh P, Zimmerman RH (eds) Micropropagation. Springer, The Netherlands, pp. 71–93

Quaintance AL, Brues CT (1905) The cotton bollworm: Bureau of entomology bulletin (No. 50). U.S. Department of Agriculture, Washington

Raubenheimer D (1992) Tannic acid, protein, and digestible carbohydrate: dietary imbalance and nutritional compensation in locusts. Ecology 73:1012–1027

Raubenheimer D, Simpson SJ (1997) Integrative models of nutrient balancing: application to insects and vertebrates. Nutr Res 10:151–179

Roeder KA, Behmer ST (2014) Lifetime consequences of food protein-carbohydrate content for an insect herbivore. Funct Ecol 28:1135–1143

Sánchez E, Rivero RM, Ruiz JM, Romero L (2004) Changes in biomass, enzymatic activity and protein concentration in roots and leaves of green bean plants (*Phaseolus vulgaris* L. cv. Strike) under high NH4NO3 application rates. Sci Hortic-Amsterdam 99:237–248

Showler AT, Moran PJ (2003) Effects of drought stressed cotton, *Gossypium hirsutum* L., on beet armyworm, *Spodoptera exigua* (Hübner), oviposition, and larval feeding preferences and growth. J Chem Ecol 29:1997–2011

Simpson SJ, Raubenheimer D (1993) A multi-level analysis of feeding behaviour: The geometry of nutritional decisions. Philos T Roy Soc B 342:381–402

Simpson SJ, Raubenheimer D (1995) The geometric analysis of feeding and nutrition: a user's guide. J Insect Physiol 41:545–553

Simpson SJ, Raubenheimer D (2001) The geometric analysis of nutrient-allelochemical interactions: a case study using locusts. Ecology 82:422–439

Simpson SJ, Raubenheimer D (2012) The nature of nutrition: a unifying framework from animal adaptation to human obesity. Princeton University Press, Princeton, NJ

Simpson SJ, Raubenheimer D, Behmer ST, Whitworth A, Wright GA (2002) A comparison of nutritional regulation in solitarious- and gregarious-phase nymphs of the desert locust *Schistocerca gregaria*. J Exp Biol 205:121–129

Simpson SJ, Sibly RM, Lee KP, Behmer ST, Raubenheimer D (2004) Optimal foraging when regulating intake of multiple nutrients. Anim Behav 68:1299–1311

Simpson SJ, Sword GA, Lorch PD, Couzin ID (2006) Cannibal crickets on a forced march for protein and salt. Proc Natl Acad Sci U S A 103:4152–4156

Singer M, Stireman J (2001) How foraging tactics determine host-plant use by a polyphagous caterpillar. Oecologia 129:98–105

Singer MS, Bernays EA, Carriere Y (2002) The interplay between nutrient balancing and toxin dilution in foraging by a generalist insect herbivore. Anim Behav 64:629–643

Snodgrass GL (1998) Distribution of the tarnished plant bug (Heteroptera: Miridae) within cotton plants. Environ Entomol 27:1089–1093

Stieger PA, Feller U (1994) Senescence and protein remobilization in leaves of maturing wheat plants grown on waterlogged soil. Plant Soil 166:173–179

Warbrick-Smith J, Behmer ST, Lee KP, Raubenheimer D, Simpson SJ (2006) Evolving resistance to obesity in an insect. Proc Natl Acad Sci U S A 103:14045–14049

Warbrick-Smith J, Raubenheimer D, Simpson SJ, Behmer ST (2009) Three hundred and fifty generations of extreme food specialization: testing predictions of nutritional ecology. Entomol Exp Appl 132:65–75

Wilkinson TL, Douglas AE (2003) Phloem amino acids and the host plant range of the polyphagous aphid, *Aphis fabae*. Entomol Exp Appl 106:103–113

Wilson LT, Waite GK (1982) Feeding pattern of Australian *Heliothis* on cotton. Environ Entomol 11(2):297–300