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Physiological factors affecting the rapid decrease in protein assimilation efficiency by a caterpillar on newly-mature tree leaves

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Abstract. *Lymantria dispar* L. caterpillars have a decreased ability to assimilate protein from mature leaves of red oak (*Quercus rubra*) compared with young, expanding leaves. The present study determines whether the drop in protein assimilation efficiency (PAE) occurs during the rapid phase of leaf maturation. Several mechanisms that might account for decreased PAE are also examined: mature leaf tissues could resist being chewed efficiently, protein in mature leaf tissues could become difficult to extract, and other nutrients in mature leaves might become growth limiting. The entire seasonal decrease in PAE occurs rapidly (in less than 2 weeks), when the leaves finished expanding. The maturation process is characterized by increased levels of fibre and decreased levels of water but no significant changes in the levels of protein or carbohydrates. Despite increased fibre in mature leaves, they are not chewed into larger food particles than are immature leaves. Carbohydrate assimilation efficiencies remain high on mature leaves, and signs of limiting water levels in larvae of *L. dispar* on mature leaves are not observed. The most important finding in the present study is the decreased extractability of protein in food particles from mature leaves, which plays a major role in explaining the rapid decrease in PAE. It is hypothesized that structural changes in cell walls during the rapid process of leaf maturation decrease protein extractability, which, in turn, greatly decreases the nutritional quality of mature oak leaves for caterpillars. The results of the present study therefore suggest a general mechanism to help explain the widely documented decrease in the nutritional quality of the mature leaves of many tree species for herbivorous insects.

Key words. Amino acid, digestion, herbivore, *Lymantria dispar*, nutritional ecology.

Introduction

One of the most striking developmental changes in the foliage of temperate deciduous trees is the shift from lush, light green expanding leaves to tough, darker green mature leaves in the spring. This rapid process appears to be triggered when leaves reach their full size. The nutritional suitability of tree leaves for caterpillars decreases greatly as the leaves mature (Hough & Pimentel, 1978; Schweitzer, 1979; Raupp & Denno, 1983; Schroeder, 1986; Raupp *et al.*, 1988; Hunter & Lechowicz, 1992; Parry *et al.*, 1998). For example, in as little as 1 week, the survivorship and growth rates of larvae of *Lymantria dispar* L. on four tree species are significantly decreased by leaf maturation (Raupp *et al.*, 1988). In a seminal paper, Feeny (1970) also notes that 'some change or combination of changes occurs in oak leaves over a period of only 2–3 weeks in late May which has a markedly adverse effect on the larval growth

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rates, pupal weights, and adult emergence of the winter moth.' Feeny (1970), and subsequent researchers, find that expanding tree leaves are rich in nitrogen and water, and have low levels of fibre and toughness, each of which could provide nutritional benefits (Scriber & Slansky, 1981).

Protein (or nitrogen) is often considered to be the most limiting macronutrient for caterpillars (Mattson, 1980; Coley *et al.*, 2006). Thus, it is logical to postulate that the acquisition of protein by caterpillars would be lower on mature foliage either because of lower foliar protein levels, decreased availability, or both. Previously, protein assimilation efficiency (PAE) was hypothesized to be decreased by increased levels of tannins or fibre. For example, increased levels of condensed tannins were believed to decrease the availability of protein in tree leaves to caterpillars (Feeny, 1970). Subsequently, it has been shown that tannins do not decrease protein assimilation by insect herbivores (Bernays, 1978; Martin *et al.*, 1987; Karowe, 1989; Barbehenn *et al.*, 2009a). It is also proposed that protease inhibitors might decrease PAE, although this is highly unlikely in mature leaves because higher levels of protease inhibitors occur in immature than in mature leaves (Cipollini & Bergelson, 2000; Haruta *et al.*, 2001). We are unaware of other chemical defences in trees that may interfere with protein assimilation to a greater extent in mature than immature leaves.

The protein : carbohydrate ratio is also considered to be a key determinant of nutritional quality (Behmer, 2009). On artificial diets, a variety of caterpillar and grasshopper species adjust their intake and/or metabolism of nutrients to obtain a specific nutrient balance. If *L. dispar* larvae that fed on mature leaves were unable to obtain sufficient levels of carbohydrates compared with those available from young leaves, it is possible that they would decrease their PAEs to optimize their protein/carbohydrate balance (Clissold *et al.*, 2010). We are unaware of any previous work that has examined this question with caterpillars on leaves.

Leaf tissues complicate the abilities of caterpillars to acquire nutrients because of the limitations imposed by leaf tissue structure. Fibre, as a structural component of cell walls and a source of toughness, decreases PAE in locusts compared with wall-free leaf preparations (Clissold *et al.*, 2009). However, it remains unclear whether PAE in insect herbivores is affected adversely by intermediate levels of fibre, such as the levels in mature versus immature leaves. Increased levels of fibre in mature leaves do have the obvious effect of increasing leaf toughness, which is associated with a decreased consumption rate (Choong, 1996). In addition, it is possible that PAE might be decreased in caterpillars feeding on mature leaves because the greater toughness of these leaves may result in the formation of larger food particle sizes, which would leave a larger fraction of cells intact and increase the diffusion pathway of protein to the gut fluid. The present study tests this possibility.

The suggestion that lower levels of water in mature foliage might explain reduced protein utilization is based, in large part, upon the finding that low water levels can become the growth-limiting nutrient in artificial diet (Martin & Van't Hof, 1988). Water is a necessary nutrient for the hydration of growing insect tissues (Martin & Van't Hof, 1988). When water limits growth, a decreased fraction of the assimilated amino acids is used for protein synthesis, and the excess amino acids are commonly excreted as waste nitrogen products (Horie & Watanabe, 1983; Martin & Van't Hof, 1988). However, artificial agar-based diets are an unsatisfactory model system for mature foliage because they lack the structural features of leaf tissues and are not difficult to chew. In the present study, experiments are conducted in a way that tests whether limiting levels of water could help explain decreased protein assimilation in larvae on mature leaves.

For the study of mechanisms that might explain the reduced nutritive value of mature foliage, it is important to distinguish between three sequential processes: protein digestion, protein assimilation and protein utilization. Digestion refers to the enzymatic hydrolysis of proteins extracted from leaf tissues. Assimilation is the absorption from the midgut of amino acids (and small peptides) derived from the digestion of proteins. Utilization is the increase in body mass as a result of protein synthesis using amino acids that have been assimilated into the haemocoel from the midgut. Thus, protein could be extracted from leaf tissues and assimilated efficiently but 'dumped' as waste nitrogen, such as when water becomes limiting. Alternatively, protein could potentially be extracted from leaf tissues but not be digested or assimilated (producing a low PAE). Such distinctions are important in the present study to understand the potential role of leaf tissue structure and processing on PAE. [Note that the term 'protein utilization efficiency' (Barbehenn *et al.*, 2013a) is no longer used synonymously with PAE.]

The insect–plant system used to examine factors that might affect PAE is *L. dispar* L. (gypsy moth) and *Quercus rubra* L. (red oak). Red oak is among the most favourable of a few hundred tree species used by *L. dispar* (Montgomery, 1990; Liebhold *et al.*, 1995). In southeastern Michigan, red oak leaves contain relatively low levels of oxidatively-active phenolic compounds, and no significant increases in either phenolic compounds or toxicity are found as red oak leaves mature (Barbehenn *et al.*, 2008, 2013a). Oak protein quality (i.e. the balance of essential amino acids) remains similar in immature and mature leaves (Barbehenn *et al.*, 2013b). However, *L. dispar* larvae grow at over twice the rate and have a two-fold greater protein assimilation rate (PAR) on immature versus mature leaves (Barbehenn *et al.*, 2013a). The purpose of the present study is to examine three main factors that might decrease PAE from mature tree leaves by *L. dispar* caterpillars: leaf chewing efficiency, protein extractability and growth-limiting levels of foliar carbohydrates or water.

To document the changes in nutritional quality that occur during leaf maturation, measurements are made of total protein, carbohydrate, water and fibre levels. To examine whether mature leaves decrease the efficiency of chewing, food particle sizes are measured in the guts of larvae that have fed on young or mature leaves. To examine whether changes in mature leaf tissues decrease the extractability of protein, the amounts of protein remaining in food particles in the guts and frass of larvae that have fed on young or mature leaves are compared. To test the hypothesis that PAE decreases as a result of decreased carbohydrate assimilation from mature leaves,

carbohydrate assimilation efficiencies of larvae on young and mature leaves are compared. To examine whether water might become limiting, an investigation of whether assimilated amino acids are metabolized and excreted as increased levels of waste nitrogen by larvae on mature (low water) leaves is performed. To examine the possibility that larvae extract and digest protein efficiently but avoid absorbing the excess amino acids from the gut, an examination is made as to whether there is an increase in free amino acids in the frass of larvae on mature leaves. Based on the results obtained, a general mechanism is proposed to help explain the reduced nutritional value of mature oak foliage.

Materials and methods

Insects

Lymantria dispar eggs were obtained from the United States Department of Agriculture (USDA, Otis Air Force Base, Massachusetts). Larvae were reared primarily at 23 °C (LD 16 : 8 h) on an artificial diet (Addy, 1969) that was modified by using linseed oil instead of wheat germ oil. Larvae were hatched at three separate times to produce experimental insects. Newly-moulted fifth-instar larvae were switched to oak leaves for each experiment.

Plants

Red oak trees ($n = 3$) that had been used in previous experiments (Barbehenn *et al.*, 2009b) were used as a source of leaves for each experiment. These trees were used in early and late May based on the appearance of their leaves; in early May they were light green and had a soft texture, whereas, in late May, they had become dark green and stiff. Leaves were removed from branches and their petioles were placed immediately in tubes of water. Within 30 min, they were washed under water with a sponge before cutting them into 2.5-cm diameter disks, avoiding the midrib. The use of leaf disks permitted accurate measurements of consumption by providing amounts of food that would be largely consumed (Schmidt & Reese, 1986). Leaf disks, equally representing the three trees, were mixed to eliminate potential between-tree variation from each experiment.

Foliar nutritional quality

Protein was measured with high performance liquid chromatography (HPLC), as total amino acids (peptide-bound plus free). Frozen (−80 °C), lyophilized samples were ground to a powder with a Retsch MM 301 mixer mill (Verder Scientific Inc., Newtown, Pennsylvania), weighed to the nearest 0.01 mg on a Cahn Electrobalance (Thermo Fisher Scientific Inc., Waltham, Massachusetts), and placed into pressure-resistant vials (Pierce, Rockford, Illinois; 7 mL). Samples (2–4 mg) were hydrolyzed in 6 M HCl (0.625 mL, oxygen free) under a nitrogen atmosphere (110 °C for 24 h). After cooling, the pH of each hydrolysate was adjusted with 6 M NaOH to fall within the range of 3–5. The derivatizing agent 6-aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was synthesized as described by Cohen & Michaud (1983). An aliquot (50 μL) of 'neutralized' hydrolysate was mixed with 50 μL of 0.5 M borate buffer (pH 8.8) and derivatized with 26.4 μL of a 10 mM AQC solution in acetonitrile. Samples were filtered and the derivatives were separated and quantified with a Waters HPLC (2690 Separation Module; Waters Corp, Milford, Massachusetts), using a Supelco Discovery HS C18 column (5.0 μm, 250 mm × 4.6 mm) with a C18 guard column. The gradient used was initially 100% A (pH 5.05 acetate buffer)/0% B (60% aqueous acetonitrile), increasing to 2% B at 0.5 min, 7% B at 10 min, 45% B at 48 min and returning to 0% B at 53 min. The flow rate was 1.0 mL min^{-1} and the column temperature was 35 °C. Peaks were detected with a Waters 996 photodiode array detector at the wavelength of maximum absorbance (248 nm). Total amino acids were quantified excluding the ammonia peak because ammonia is produced by the deamination of certain amino acids during hydrolysis, as well as by *L. dispar* larvae, both of which would confound measurements of PAE. Standard curves were prepared from hydrolyzed tobacco leaf protein (fraction 1; primarily RuBisCO; provided by M. M. Martin, University of Michigan) as a representative foliar protein. Amino acid composition and protein quantity remained stable in oak leaf disks during 1-day feeding periods (Barbehenn *et al.*, 2013b).

Water was measured in each of the feeding experiments by weighing leaf disks before and after they were oven dried (70 °C).

Sugars were measured with an enzymatic method (Zhao *et al.*, 2010). Briefly, ground lyophilized leaf samples (10 mg) and frass samples (20 mg) were extracted with 80% ethanol (600 μL, 30 min at 45 °C) with shaking, followed by centrifugation (8000 ***g*** for 5 min). The supernatant solutions from three extractions were pooled, and then clarified with activated charcoal (50 mg for 10 min), followed by centrifugation (8000 ***g*** for 5 min). Aliquots of the solutions (20 μL from leaves, 40 μL from frass) were dried in a 96-well microplate (60 °C for 45 min). Dried samples and standards were resolubilized in 40 μL of double-distilled water, to which 100 μL of glucose assay reagent (Sigma Chemical Co., St Louis, Missouri; G3293) was added. Total sugars were measured as glucose by adding 20 μL aliquots of invertase (for hydrolyzing sucrose) and phosphoglucose isomerase (for converting fructose to glucose) to each sample well (Zhao *et al.*, 2010). Plates were mixed, incubated for 60 min (37 °C), and the absorbance of each well was recorded at 340 nm. Sample blanks were prepared to correct for the absorbance of interfering compounds. Glucose standards (0–15 μg) were run on each microplate to produce standard curves for quantifying total sugars in the samples. Sucrose and fructose standards were run to confirm that they were quantitatively converted to glucose in the assay conditions. No correction for the mass of water gained during sucrose hydrolysis was made, thus overestimating this sugar by 4%. Preliminary measurements of free glucose, fructose and sucrose concentrations in leaves indicated that they were present in a ratio of 1 : 1.5 : 3.

Starch was measured in the extracted pellets remaining from leaf and frass samples (Zhao *et al.*, 2010). After the addition of 1 mL of double-distilled water to each tube, the pellets were boiled for 1 h. Starch was then converted to glucose by amylase and amyloglucosidase. Glucose liberated from starch was measured with the glucose assay reagent, as described above. Sample blanks were prepared to correct for the absorbance of interfering compounds. The mass of glucose was corrected ($\times 0.90$) to account for the mass of water gained during starch hydrolysis. High starch levels measured in the frass of some larvae was confirmed by repeated analysis. All reagents were purchased from Sigma Chemical Co.

Total fibre was measured with the neutral detergent fibre (NDF) assay, modified from Van Soest & Wine (1967) by eliminating the use of decahydronaphthalene, sodium sulphite, and the acetone wash. Ground samples (40 mg) were analyzed from three replicate dates in each experiment. Samples were placed in weighed 2-mL plastic centrifuge tubes with screw-cap lids. Samples were extracted in the neutral detergent solution (1.5 mL) at 100 °C for 1 h. After cooling and centrifugation (8000 ***g*** for 5 min), the supernatant solutions were removed. The extraction was repeated. Fibre pellets were dispersed in distilled water (1.5 mL), and washed free of detergent in a shaker for 30 min (ambient temperature). The fibre was pelleted by centrifugation. Supernatant solutions were discarded, and the rinsing procedure was repeated two more times. Fibre samples were dried (70 °C) for 24 h in the open-topped tubes. The tube and fibre were weighed together, and the weight of the fibre was determined by subtracting the tube weight from the total weight. The percentage of fibre was calculated as (final dry weight)/(initial dry weight) $\times$ 100. Control tubes ($n = 3$) taken through the entire procedure were used to correct for the mass lost from the tubes (< 1 mg). Neutral detergent fibre includes cellulose, hemicellulose and lignin but not pectin. To distinguish cellulose, hemicellulose and lignin, the NDF assay was followed on the same samples by the acid detergent fibre (ADF) assay and then an assay for lignin.

The ADF solution was prepared by dissolving 1 g hexadecyltrimethylammonium bromide in 97.2 mL of distilled water, containing 2.8 mL of sulphuric acid. The ADF solution (1.5 mL) was added to NDF pellets in their original tubes, which were boiled for 1 h. The pellets remaining after centrifugation (8000 ***g*** for 5 min) were extracted a second time, after which the pellets were washed three times with 1.5 mL of distilled water (30 min, ambient temperature with shaking). Fibre samples were dried (70 °C) for 24 h in the open-topped tubes, and the remaining sample weights were determined by subtracting the tube weights.

Lignin (Klason) was determined by treating the ADF pellets with 72% sulphuric acid (Dence, 1992). Briefly, pellets were shaken for 1 h (30 °C) in their original tubes. The entire contents of the tubes were transferred to 15-mL centrifuge tubes (plug-seal screw cap; Corning Inc., Lowell, Massachusetts), after which the sulphuric acid was diluted 1 : 12 with double-distilled water. Diluted samples were boiled for 4 h. After further dilution to a total of 14.5 mL, the samples were centrifuged (2000 ***g*** for 15 min). Supernatant solutions were largely discarded, and the lignin pellets were resuspended and returned to the original tubes for washing and drying, as described above. Cellulose was determined as the difference between ADF and lignin. Hemicellulose was determined as the difference between NDF and ADF.

Food particle size

Food particles were dissected from the foreguts of fifth-instar larvae on 9 May, 25 May and 27 June 2012 after a 2-day period of feeding on leaf disks ($n = 12–15$ larvae per date). The particles were dispersed in 500 μL of 50% methanol and stored at −80 °C. Samples were thawed, centrifuged (8000 ***g*** for 3 min), and the supernatant solutions were removed. To increase visual contrast, particles were stained with toluidine blue O (30 μL, 0.01% in water for 1 min). Stained particles were mixed with 500 μL of water to rinse excess stain, centrifuged and the supernatant solutions were removed. Stained particles were resuspended in 500 μL of water, and a 40-μL aliquot was placed on a microscope slide with a cover slip. Photographs were taken with a Leica stereomicroscope ($\times 10$ magnification). All particles greater than 0.1 μm^2 were measured using IMAGE PRO PLUS (MediaCybernetics Inc., Rockville, Maryland). Data were exported to EXCEL (Microsoft, Redmond, California) for graphical analysis, using bin sizes of 10 μm^2. Occasional particles greater than 0.37 mm^2 were not included in the analysis.

Protein assimilation efficiency

Newly-moulted fifth-instar larvae were fed oak leaves for a 3-day period in experiments that were begun on 7 May, 22 May and 25 June 2012. Larvae ($n = 15$ per experiment) were kept in 35-mL snap-cap plastic cups. Groups of three to seven freshly cut leaf disks were weighed for each larva daily. A moistened paper filter was placed in the bottom of each cup, and hydrated daily, to keep the leaf disks turgid. Uneaten food was dried (70 °C) each day to determine the amount eaten. Representative leaf disks were weighed fresh and after drying to estimate the dry weight of food given to each larva on each day. Additional leaf disks were frozen (−80 °C) daily and lyophilized. Frass was collected daily, and kept separately for each larva in screw-cap centrifuge tubes at −80 °C. After 3 days of feeding, larvae were starved for a 3-h period and then fed a piece of artificial diet. All remaining frass pellets composed of leaf material were combined with the frass for each caterpillar, frozen and lyophilized. Protein was measured as total amino acids (peptide bound + free) in HCl hydrolysates with HPLC, as described above. The nutritional indices examined for protein were:

Protein consumption rate (PCR) = mass of protein ingested/day

Protein assimilation efficiency (PAE) = (mass of protein ingested – mass of protein egested)/mass of protein ingested $\times$ 100

Protein assimilation rate (PAR) = (mass of protein ingested – mass of protein egested)/day

PAE is identical to the 'approximate digestibility' of protein. PAE is 'approximate' as a result of the inclusion of nonplant protein in the frass (e.g. protein from peritrophic membranes and sloughed midgut cells). Protein ingested was calculated as percent protein in leaves × total mass ingested, and total protein egested was calculated as percent protein in frass × total mass egested.

Carbohydrate assimilation efficiencies and assimilation rates were measured in the same manner as described for protein by analyzing sugar and starch levels in the food and frass of ten larvae used for measuring PAE.

Protein levels in food particles

Protein remaining inside ingested leaf particles was measured from samples in the posterior midgut. This method permitted the surrounding gut fluid (containing free protein and amino acids) to be quickly rinsed from the food particles. Fifth-instar larvae ($n = 12–15$ per date) were fed for a 2-day period on disks from immature leaves (beginning 9 May) or on disks from mature leaves (beginning on May 25 or June 27). Larvae were chilled individually (6 min at −20 °C), and dissected to remove the entire gut. The contents of the posterior midgut were placed in 500 μL of 20 mM HCl (ambient oxygen), dispersed by shaking, and immediately centrifuged (8000 ***g*** for 3 min). Supernatant solutions were removed and the pellets were lyophilized. Subsamples of the pellets were weighed and hydrolyzed in 6 M HCl. Total amino acid levels were measured with HPLC, as described above.

Waste nitrogen products

Waste nitrogen products were extracted from frass samples collected during each of the three feeding studies. Frass was lyophilized and ground to a fine powder with a MM 301 mixer mill (Retsch, Germany). Uric acid and ammonium salts were extracted from 5-mg samples in 500 μL of pH 2.1 buffer (containing 10 mg polyvinylpolypyrrolidone) at 60 °C for 30 min (Shingfield & Offer, 1999). Samples from 8 to 15 replicate larvae per treatment group were analyzed. Uric acid was measured with a Waters 2690 Separations Module HPLC, coupled with a Waters 996 photodiode array detector. Uric acid was separated with a pair of connected columns: a Polaris C18 (3.0 μm, 150 mm × 4.6 mm) (Metachem) and a Zorbax Bonus-RP C14 (3.5 μm, 150 mm × 4.6 mm) (Agilent Technologies Inc., Santa Clara, California) with a C18 guard column. A pH 3.0 buffer was used as the mobile phase (1.0 mL min^{-1}, 40 °C) (Shingfield & Offer, 1999). Uric acid was identified based on its peak retention time and ultraviolet-visible spectrum (compared with uric acid standards) and quantified with a uric acid standard curve. Ammonium salts (Lovett *et al.*, 1998) were measured in the frass extracts with the HPLC method used to measure amino acids (above). A standard curve was prepared from ammonium phosphate (Sigma Chemical Co.) by serial dilution in the pH 2.1 buffer. Two other waste nitrogen products formed by some larval Lepidoptera, allantoin and allantoic acid (Bursell, 1967), were measured with the above HPLC method but were below detectable levels in the frass of *L. dispar*.

Statistical analysis

Analyses of foliar carbohydrate, water and fibre levels, particle sizes, larval consumption rates, amounts of unextracted protein, faecal protein and waste nitrogen levels were compared between dates with one-way analysis of variance (ANOVA) (SAS Institute, 2010). The assimilation efficiencies of total dry mass, protein and carbohydrates were compared between dates with analysis of covariance, using egested masses as main factors and ingested masses as covariates. The normality of the residuals was confirmed with the Shapiro–Wilk test (PROC MIXED). Post-hoc multiple comparisons were made using the probabilities of differences between least squares means (PROC MIXED). These comparisons tested *a priori* hypotheses. Therefore, $P = 0.05$ was used to determine statistical significance. Foliar protein levels could not be transformed to meet the assumptions of ANOVA, and were analyzed with a Kruskal–Wallis test. Individual larvae were used as replicates for all experiments on insects. (For sample sizes, see Materials and methods and Results.) Replicates for oak leaf analyses were days within experiments ($n = 3$ per experiment).

Results

Foliar nutritional quality

As red oak leaves matured during a 2-week period in May, their protein levels declined by only 9% ($P = 0.121$) (Table 1). Protein levels remained at a similar level through June. By contrast, water levels declined significantly by 17% during leaf maturation, and continued to decline over the next month ($P < 0.001$ for each date) (Table 1). Leaf density increased by 74% in May, and by an additional 26% in June, consistent with increased toughness (Barbehenn *et al.*, 2013a).

Sugars comprised 73–82% of total carbohydrates (Table 2). As with protein, carbohydrates remained at similar levels throughout leaf maturation. Thus, ratios of protein :

Table 1. Protein and water levels and leaf density of immature (early May) and mature (late May and June) red oak leaves.

Date	Protein (% dry weight)	Water (% fresh weight)	Density (mg cm^{-2})
9–11 May	20.9 ± 0.5^{a}	71.9 ± 0.2^{c}	3.8 ± 0.3^{a}
25–27 May	19.1 ± 0.5^{a}	60.0 ± 1.1^{b}	6.6 ± 0.4^{b}
25–27 June	18.7 ± 1.2^{a}	54.8 ± 0.1^{a}	8.3 ± 0.6^{c}
	$P = 0.118$	$P < 0.001$	$P = 0.002$

Protein was measured as total amino acids (polypeptide-bound plus free) in acid hydolysates. Density was measured on a dry weight basis. Data are presented as the mean ± SE ($n = 3$ days/date for all measurements). Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$).

Table 2. Carbohydrate and fibre composition of immature (early May) and mature (late May and June) red oak leaves.

Date	Sugars (% DW)	Starch (% DW)	Total carbohydrate (% DW)	Cellulose (% DW)	Hemicellulose (% DW)	Lignin (% DW)	Total fibre (% DW)
9–11 May	6.0 ± 0.4^{a}	2.2 ± 0.3^{a}	8.2 ± 0.8^{a}	8.1 ± 0.7^{a}	11.4 ± 0.8^{a}	5.8 ± 0.5^{a}	25.3 ± 0.4^{a}
25–27 May	7.0 ± 0.3^{a}	2.3 ± 0.3^{a}	9.3 ± 0.3^{a}	13.3 ± 0.1^{b}	12.8 ± 1.0^{a}	9.7 ± 0.2^{b}	34.7 ± 0.8^{b}
25–27 June	7.0 ± 0.4^{a}	1.5 ± 0.5^{a}	8.5 ± 0.9^{a}	12.3 ± 0.9^{b}	12.9 ± 0.8^{a}	8.9 ± 0.6^{b}	34.0 ± 1.3^{b}
	$P = 0.242$	$P = 0.503$	$P = 0.668$	$P = 0.004$	$P = 0.464$	$P = 0.007$	$P = 0.002$

DW, dry weight. Protein was measured as total amino acids (polypeptide-bound plus free) in acid hydolysates. Data are presented the as mean ± SE ($n = 3$ replicate days/date for all measurements). Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$). Total fibre does not include pectin.

Table 3. Consumption and assimilation of immature (early May) and mature (late May and June) red oak leaves and protein by fifth-instar *Lymantria dispar* larvae.

Date	Total consumption rate (mg day^{-1})	Total assimilation efficiency (%)	Protein consumption rate (mg day^{-1})	Protein assimilation efficiency (%)	Protein assimilation rate (mg day^{-1})
9–11 May	100.5 ± 1.3^{c}	36.1 ± 0.9^{c}	21.0 ± 0.3^{c}	70.8 ± 1.0^{b}	14.9 ± 0.3^{c}
25–27 May	80.9 ± 2.1^{b}	29.2 ± 0.5^{b}	15.4 ± 0.5^{b}	47.7 ± 0.8^{a}	7.4 ± 0.2^{b}
25–27 June	68.1 ± 3.7^{a}	26.1 ± 0.8^{a}	12.7 ± 0.8^{a}	48.2 ± 1.2^{a}	6.1 ± 0.4^{a}
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Assimilation = mass ingested – mass egested. Data are presented as the mean ± SE ($n = 13–15$ for all measurements). Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$).

carbohydrate remained in the range of 2.0–2.4 in mature and young leaves, respectively. As expected, when lush oak leaves matured, total fibre increased significantly by 37% ($P = 0.002$), but no further change in fibre was observed in June. The fibre components that explained this increase were cellulose and lignin, whereas hemicellulose levels did not increase significantly (Table 2).

Protein assimilation

The protein in immature leaves was assimilated efficiently by *L. dispar* larvae but, within a period of 2 weeks, PAE dropped by 33% ($P < 0.001$) (Table 3). This lower PAE persisted into the summer. The combined effects of decreased PAE and decreased protein consumption rate cut the protein assimilation rate in half for larvae feeding on recently mature leaves ($P < 0.001$) because PAR = protein consumption rate × PAE. Total consumption rate (CR) and assimilation efficiency (AE) of larvae also decreased significantly on recently mature leaves but by approximately 19%, compared with a 27% and 33% drop in protein consumption rate and PAE, respectively. Decreased AE was consistent with increased levels of fibre (indigestible) and a decrease in protein assimilation. These results are similar to previous observations (Barbehenn *et al.*, 2013a), but they show the narrow window of time in which major changes occurred.

Carbohydrate assimilation

Sugars (glucose, fructose and sucrose) were assimilated with extremely high efficiencies (Table 4). By contrast, starch was poorly digested from leaves of all ages. Although there were statistically significant decreases in starch and total carbohydrate assimilation efficiencies from recently mature leaves, high assimilation efficiencies from fully mature summer leaves suggested that this relatively small effect was not related

Table 4. Assimilation of carbohydrates from immature (early May) and mature (late May and June) red oak leaves by fifth-instar *Lymantria dispar* larvae, as well as ratios of protein : carbohydrate assimilation rates.

Date	Sugar assimilation efficiency (%)	Starch assimilation efficiency (%)	Carbohydrate assimilation efficiency (%)	Carbohydrate assimilation rate (mg/day)	Protein AR: Carbohydrate AR ratio
9–11 May	96.1 ± 0.3^{a}	53.5 ± 3.0^{b}	84.7 ± 0.9^{b}	7.2 ± 0.2^{c}	2.05 ± 0.04^{b}
25–27 May	97.4 ± 0.2^{b}	31.9 ± 3.4^{a}	80.9 ± 0.9^{a}	6.2 ± 0.2^{b}	1.20 ± 0.03^{a}
25–27 June	97.9 ± 0.1^{b}	40.1 ± 5.0^{b}	86.3 ± 1.0^{b}	5.2 ± 0.4^{a}	1.22 ± 0.04^{a}
	$P < 0.001$	$P < 0.001$	$P = 0.001$	$P < 0.001$	$P < 0.001$

Assimilation = mass ingested – mass egested. AR, assimilation rate. Data are presented the as mean ± SE ($n = 10–15$ for all measurements). Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$).

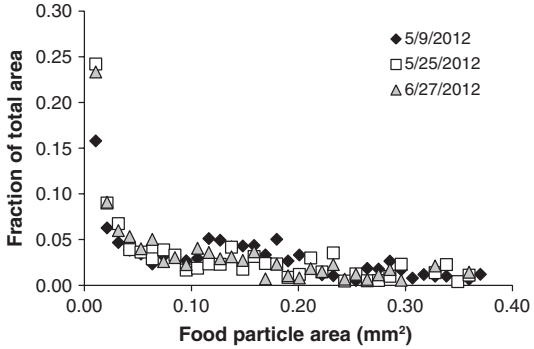

Fig. 1. Particle size distributions in the foreguts of fifth-instar *Lymantria dispar* larvae feeding on immature (9 May 2012) and mature (25 May and 27 June 2012) red oak leaves.

to leaf maturation. It is noteworthy that efficiencies and rates of carbohydrate assimilation from mature leaves did not decrease in the same manner as did PAR (e.g. only a 13% decrease in carbohydrate assimilation rate was observed from recently mature leaves). Ratios of protein : carbohydrate assimilation rates decreased by 42% when oak leaves matured, which can be ascribed largely to decreased PAE.

Food particle size

Contrary to expectation, median particle sizes produced by larvae on immature and mature leaves did not differ significantly ($P = 0.484$). Instead, larvae chewed all leaves into a wide range of particle sizes (Fig. 1), with the smallest category of particles ($0.01–1.1\,\mu m^2$) representing the highest percentage of total particle area. Larvae produced a significantly higher fraction of small particles from mature leaves than from immature leaves ($P < 0.01$ for both comparisons) (Fig. 1). Thus, higher PAE on lush leaves was not a result of the larvae chewing them into finer particles, a result that is contrary to one of the earliest mechanisms proposed to explain the reduced nutrient value of mature foliage.

Protein levels in food particles and frass

Food particles taken from the posterior midgut contained 36% higher levels of protein when larvae fed on recently mature leaves than on immature leaves (Fig. 2) ($P = 0.008$). When larvae fed on mature leaves in June, there was 49% more protein retained in the food particles ($P < 0.001$). These results were consistent with the second mechanism examined; the extractability of foliar protein decreased from mature leaves. As with food particles, protein levels in the frass were 37–48% higher from mature than immature leaves ($P < 0.001$ for all mature leaves). Therefore, the protein remaining in the frass represents the fraction that could not be extracted from the ingested leaf tissues, rather than protein that was extracted but not assimilated. It can be calculated that two-thirds of the decrease in PAE (i.e. a decrease to 56% PAE) is explained by the increase in unextractable protein in mature leaf tissues.

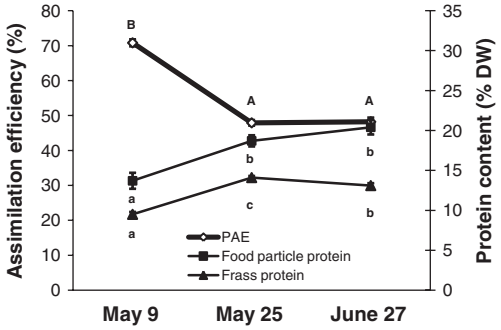

Fig. 2. Protein assimilation efficiency (PAE) of fifth-instar *Lymantria dispar* on immature and mature red oak leaves, and levels of protein remaining in food particles and frass from larvae on these leaves. Dissections to measure protein inside food particles were performed on the given dates. Separate sets of larvae were used for feeding experiments to measure PAE and frass protein, which were performed for 3-day periods beginning on 7 May, 22 May and 25 June 2012. Protein was measured as total amino acids (peptide-bound plus free) in acid hydolysates. Different letters designate significantly different means within each series ($P < 0.01$). DW, dry weight.

Table 5. Water consumption and waste nitrogen excretion by *Lymantria dispar* larvae that fed on immature (early May) or mature (late May and June) red oak leaves.

Date	Water consumption rate (mg day^{-1})	Uric acid (% DW)	Ammonium salts (% DW)	Total waste nitrogen (% DW)
9–11 May	247 ± 4^{c}	0.68 ± 0.04^{b}	0.82 ± 0.06^{c}	1.50 ± 0.09^{c}
25–27 May	122 ± 3^{b}	0.11 ± 0.01^{a}	0.27 ± 0.04^{b}	0.39 ± 0.05^{b}
25–27 June	84 ± 5^{a}	0.10 ± 0.02^{a}	0.02 ± 0.02^{a}	0.12 ± 0.02^{a}
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Data are presented as the mean ± SE ($n = 8–15$/date for all measurements). Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$).

Water consumption and waste nitrogen production

The consumption rate of water decreased by 51% from early to late May, and by 66% by late June ($P < 0.001$ for both dates) (Table 5). This decrease resulted from both decreased foliar water levels and decreased total CR (Tables 1 and 2). However, the decreased consumption of water in mature leaves was not associated with increased levels of waste nitrogen products (Table 5). Instead, the excretion of uric acid was significantly higher from larvae on immature leaves ($P < 0.001$). The excretion of ammonium salts was also significantly higher from larvae on immature (high-water) leaves, rather than on mature leaves ($P < 0.001$) and was below detection from many larvae in June. Thus, total waste nitrogen levels were positively associated with total protein assimilated ($R^2 = 0.894$, $P < 0.001$), which is the opposite of the expected pattern if low water levels limited growth, and excess protein were metabolized and excreted. In addition, only small amounts of free amino acids (i.e. 0.23 ± 0.03% dry

weight) were present in the frass of insects on immature and mature leaves (compared with over 10% dry weight total amino acids).

Discussion

As expected from previous work on the *L. dispar*-red oak system, larvae assimilate protein more efficiently from immature leaves than from mature leaves (Barbehenn *et al.*, 2013a). The present study shows that the entire seasonal decrease in PAE occurs rapidly (in less than 2 weeks) in association with the completion of leaf expansion. This rapid maturation process is characterized by increased levels of fibre and decreased levels of water but no marked changes in protein or carbohydrate levels. The main purpose of the present study was to examine why caterpillar PAE decreases in association with leaf maturation. The fact that both the rinsed food particles and frass from *L. dispar* contain increased protein levels when mature leaves are eaten points to the same major finding, that protein in food particles becomes significantly less extractable during leaf maturation.

The present results place a greater emphasis on protein extractability and less emphasis on seasonal changes in concentrations of nutrients to explain protein availability. The major portion of the drop in nitrogen levels in tree leaves commonly occurs from the time of bud break through the production of primordial leaves (which are rich in nucleic acid nitrogen). After leaf expansion nears completion, there is commonly little change in protein levels (Feeny, 1970; Schroeder & Malmer, 1980; Wint, 1981; Williams *et al.*, 1998; Barbehenn *et al.*, 2013a; present study; but see Coley *et al.*, 2006). It is noteworthy that the range of protein found in red oak leaves during this study coincides with the optimum range selected by *L. dispar* larvae from artificial diets (Stockoff, 1993).

High assimilation efficiencies for sugars (e.g. $> 90\%$) are reported in previous studies of caterpillars on foliage (Horie *et al.*, 1985; Terra *et al.*, 1987). However, starch granules *in planta* are digested with widely variable and sometimes negligible efficiencies by different species of caterpillars (Waldbauer, 1968; Harvey, 1975). Low efficiencies of starch assimilation could be related to the inability of starch to diffuse through cell walls, in contrast to small molecules such as sugars (Baron-Epel *et al.*, 1988). However, starch assimilation efficiencies have only small effects on total carbohydrate assimilation rates because sugars are assimilated efficiently and comprise the major form of carbohydrate in red oak leaves. Importantly, little evidence is found to support the hypothesis that PAE decreases as a result of decreased carbohydrate assimilation efficiency from mature leaves. In addition, neither consumption rates, nor growth rates of *L. dispar* larvae increase significantly when sucrose or fructose is coated on mature red oak leaves at 1.5% of leaf dry weight (R. V. Barbehenn, unpublished data), which is inconsistent with carbohydrate limitation.

Some studies on protein utilization conclude that the low levels of water present in mature tree leaves limit caterpillar growth, and consequently reduce their utilization of protein (Scriber, 1977, 1979; Martin & Van't Hof, 1988). Therefore, two potential post-ingestive responses of water-limited caterpillars would be the increased production of waste nitrogen compounds (from metabolized excess amino acids) and/or the intentional decrease of PAE (reducing the costs of waste nitrogen production). The present results do not support these physiological scenarios. First, larvae that fed on mature leaves do not produce higher levels of waste nitrogen compounds. Instead, waste nitrogen levels are much higher on lush, young leaves. This is interpreted as a result of the mismatch between the balance of amino acids in foliar protein and the balance required for larval protein synthesis (Barbehenn *et al.*, 2013b) (i.e. unneeded amino acids will end up excreted as waste nitrogen in proportion to the amount of foliar protein used for growth). Most importantly, protein is physically trapped in larger amounts in mature than in young leaf particles ingested by larvae. Thus, protein is not extracted to a similar extent from all leaf ages, and absorbed from the midgut to a lesser extent by larvae on mature leaves. Other signs of water limitation observed previously included decreased efficiencies of converting assimilated matter to body mass (ECD) (Scriber, 1977; Martin & Van't Hof, 1988). It is noteworthy, therefore, that no significant changes in ECD are observed in *L. dispar* larvae on mature versus immature oak leaves (Barbehenn *et al.*, 2013a). The different results obtained in the present study compared to previous work are likely a result of the structural effects of leaf tissues on protein availability versus artificial diets (Martin & Van't Hof, 1988), and the smaller range of water levels in maturing oak leaves (72% to 60%) compared with other work conducted with wilted leaves (e.g. 90% to 50%) (Scriber, 1977). Based on this evidence, lower water consumption rates by *L. dispar* larvae on mature leaves do not appear to explain decreased PAE from mature oak leaves.

The structural and morphological changes that occur during the rapid maturation of leaves are well studied (Maksymowych, 1973; Dale, 1982) but not with respect to their potential effects on protein assimilation by insect herbivores. Increased fibre in mature leaves is associated with increased leaf toughness and decreased consumption rate (Choong, 1996; Barbehenn *et al.*, 2013a) but leaf tissue processing is no less efficient by *L. dispar* larvae on mature than on immature leaves. Nonetheless, fibre might still affect PAE. The cell walls in immature leaves are thin (approximately 0.1 μm) (Doblin *et al.*, 2003). By contrast, mature cell walls are greatly thickened with fibre (up to several μm thick) and contain increased proportions of lignin, pectin and cell wall proteins. Some of these changes are evident in the oak leaves used in the present study, and any of these changes could potentially decrease protein extractability. For example, increased amounts of lignin in cell walls greatly increase leaf toughness, and also decrease the porosity of the cell wall (adding 'water-proofing') (Boudet, 2003). Pectin is considered to have strong effects on the permeability properties of cell walls (Baron-Epel *et al.*, 1988; but see Fujino & Itoh, 1998). Cell wall-bound protein also decreases the permeability of cell walls and increases their strength (Brisson *et al.*, 1994). Bound protein increases in red oak during leaf maturation

from 4% to 11% of total protein (Barbehenn *et al.*, 2013b). It can be calculated, however, that the increase in cell wall-bound protein makes only a trivial (< 4%) contribution to the overall drop in PAE. Unexpectedly, condensed tannins in red oak cell walls do not increase significantly during leaf maturation (Barbehenn *et al.*, 2013a). Cell membranes are also unlikely to limit protein extraction because membranes are degraded rapidly in the guts of caterpillars (Barbehenn, 1992). Presumably, protein extraction is a passive process that follows the disruption of cell membranes and the exposure of leaf tissues to the highly basic pH of caterpillar midgut fluid (Schultz & Lechowicz, 1986). The importance of cell wall structure is also suggested in the seminal study of Feeny (1970), in which it is shown that grinding the leaves of young and mature *Quercus robur* eliminates their nutritional differences for a caterpillar.

Although the mandibles of *L. dispar* caterpillars lack a true molar region or an array of non-incisor teeth (Bernays & Janzen, 1988), they are able to chew leaves efficiently. The present study provides the first evidence, of which we are aware, regarding such a capability. Chewing is apparently accomplished when one mandible crushes an excised leaf piece into a recessed groove in the opposing mandible (Peterson, 1951: fig. L24 E). *Lymantria dispar* produces food particles with an array of sizes on par with those produced by grasshoppers, which have true molars (Barbehenn, 2005). Surprisingly, caterpillars with chewing mandibles represent less than 20% of 202 species of Lepidoptera surveyed (Barbehenn, 1992), making snipping mandibles (which produce large food particles) the predominant morphology. Therefore, small particle size may not be the key to extracting foliar protein. Further work is needed to understand whether *L. dispar* extracts a nutritionally significant fraction of foliar protein through the cell walls of intact leaf cells, as do leaf-snipping caterpillars. This evidence would be relevant to the hypothesis that cell wall maturation decreases PAE by decreasing protein extractability.

The results of the present study contribute to an understanding of the nutritional factors that decrease the fitness of *L. dispar* (and possibly other spring-feeding species) when they feed on recently mature tree leaves (Hough & Pimentel, 1978; Schweitzer, 1979; Schroeder, 1986; Raupp *et al.*, 1988; Parry *et al.*, 1998). Specifically, late-hatching larvae may face a greatly reduced ability to acquire protein if they must feed on mature leaves for a greater part of their developmental period. Any factor that limits protein assimilation might be expected to have a strong impact on caterpillar fitness. The fact that PAE declines in a similar fashion from the mature leaves of both red oak and sugar maple (Barbehenn *et al.*, 2013a) suggests that this phenomenon spans a wide taxonomic range of plants. Thus, high PAE from immature leaves, if it is a general phenomenon, would have potentially important ecological and evolutionary consequences. For example, the nutritional benefits of immature leaves to herbivores, and the high cost to plants from damage to these leaves, may have contributed to the large investment in plant defences commonly found at this stage of development (Herms & Mattson, 1992; Haruta *et al.*, 2001; Coley *et al.*, 2006).

In conclusion, although protein remains near optimum levels for *L. dispar* in mature oak leaves, the amounts that could be assimilated from these leaves fell to approximately one half of this level. The results of the present study therefore suggest a general mechanism to help explain the widely documented decrease in the nutritional quality of the mature leaves of many tree species for herbivorous insects: lower protein extractability decreases PAE. Although carbohydrates and water are also essential nutrients, variation in their levels does not explain decreased PAE or consumption rate in larvae on mature oak leaves. It is hypothesized that the major changes in cell wall structure during leaf maturation are primarily responsible for decreasing PAE. Support for this hypothesis would provide a 'next step' to help explain the reduced nutritional quality of the mature leaves of some tree species for caterpillars.

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