Effects of leaf maturity and wind stress on the nutrition of a generalist caterpillar, *Lymantria dispar*, feeding on poplar

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Running title: *Effects of leaf maturity and wind on herbivores*

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Abstract. The growth rates of insect herbivores commonly decrease when they feed on mature leaves, a result of the combined effects of several nutritional and physiological mechanisms. Environmental stresses during leaf development may also decrease herbivore performance. This study tests two main hypotheses to help clarify the importance of these factors for the nutrition and growth of an insect herbivore: (i) that decreases in nutrient levels, consumption rates, and nutrient assimilation efficiencies impact negatively on herbivores feeding on mature leaves, and (ii) that wind stress has a negative impact on herbivores feeding on mature leaves. The results show that mature poplar (*Populus alba* x *P. tremula*) leaves have decreased levels of protein and increased levels of fibre, and that growth rates of gypsy moth (*Lymantria dispar* L.) are decreased on mature leaves in association with decreased consumption rates. However, contrary to the first hypothesis, protein and carbohydrate are assimilated efficiently (74-82% and 84-87%, respectively) from immature and mature poplar leaves. The larvae are able to chew mature leaves as efficiently as immature leaves, potentially maximizing nutrient extraction. Contrary to the second hypothesis, wind-stressed leaves have no significant detrimental effects on nutrient assimilation efficiencies, and the lower growth rates of *L. dispar* larvae feeding on mature wind-stressed leaves can be explained by lower consumption rates. Therefore, the availability of nutrients to herbivores feeding on mature tree leaves is not necessarily impacted by lower assimilation efficiencies, even when leaves develop under wind stress. These results help explain some of the large variation between the nutritional qualities of trees for forest Lepidoptera.

Key words: assimilation, carbohydrates, digestion, insect herbivore, larva, nutrients, protein,

Introduction

The nutritional quality of plants has a major impact on the fitness of insect herbivores. Caterpillar growth rate and body mass (fitness-related parameters) are generally greatest for larvae that feed on immature leaves (Feeny, 1970; Hough & Pimentel, 1978; Schweitzer, 1979; Raupp & Denno, 1983; Raupp *et al.*, 1988; Schroeder, 1986; Hunter & Lechowicz, 1992; Parry *et al.,* 1998; Kursar & Coley, 2003).

As leaves mature, their nutritional quality typically declines because of the lower availability of some nutrients and increased fibre and toughness. Decreased levels of foliar protein, carbohydrates, and/or water in mature tree leaves can each impact negatively on the growth rates of insects (Scriber, 1979; Mattson, 1980; Coley *et al.,* 2006). Nutrient availability is also affected by how efficiently nutrients are extracted, digested and absorbed (or "assimilated") by herbivores. Increased fibre is associated with increased leaf toughness, which commonly decreases the consumption rates of insect herbivores (Kursar & Coley, 2003, Clissold *et al.,* 2009). In turn, decreased nutrient assimilation rates have a negative impact on herbivore growth rates.

Abiotic stresses, such as wind, during leaf development can cause plant responses that potentially decrease foliar nutritional quality for insect herbivores. Wind stress on some plants causes them to produce more flexible and fibrous leaves, and to develop foliar characteristics that minimize water loss from transpiration (Jaffe & Forbes, 1993; Anten *et al.*, 2010). A major study on the effects of wind stress on leaf composition shows increased levels of lignin and peroxidase activity in stressed bean leaves (Cipollini, 1997). However, few studies have

examined foliar nutritional components other than fibre. Under certain conditions, the mechanical stimulation of a grass lowers foliar nitrogen levels significantly, but does not consistently affect carbohydrates (Kraus *et al.*, 1994). To characterize the effects of a major abiotic stress on foliar nutritional quality better, this study examines the effects of wind stress on foliar fibre components, as well as nutrients such as protein and carbohydrate.

No previous work, of which the authors are aware, examines the impact of wind stress on the nutritional physiology of insect herbivores. Previous work on mite populations shows that wind-stressed bean leaves support lower reproductive rates (Cipollini, 1997). However, wind-stressed tomato leaves have no significant effect on the growth rates of *Manduca sexta* caterpillars (Cipollini & Redman, 1999). In these cases, it is unclear whether the herbivores are affected because of decreased feeding (mites), or are unaffected because of compensatory responses, such as increased consumption rates (*M. sexta*). A major purpose of this study is to examine the mechanisms underlying the impact of wind stress on the nutrition of a tree leaf-feeding insect.

The availability of nutrients such as protein can decline from mature leaves not only because of a decrease in their levels, but also because of decreased extractability. Decreased protein extractability is largely responsible for the decline in protein assimilation efficiency (PAE) by *Lymantria dispar* L. larvae on the mature leaves of red oak (*Quercus rubra*) and presumably also on sugar maple (*Acer saccharum*) (Barbehenn *et al.*, 2013a, 2014). Caterpillars feeding on lush, immature oak and maple leaves assimilate protein with efficiencies of 70-80%, which decline after leaf maturation to efficiencies of less than 50%. By contrast, when *L. dispar*

larvae feed on several species in the willow family (Salicaceae), there appears to be little decrease in protein or carbohydrate assimilation efficiencies (Barbehenn *et al.*, 2015). In this study, it is expected that PAE remains high in *L. dispar* that feed on the mature leaves of hybrid poplar (*Populus alba* x *P. tremula*), and that high PAE is associated with high protein extractability from ingested leaf tissues. Previous work on carbohydrate assimilation efficiency (CAE) shows that sugar assimilation efficiencies remain high (ca. 90%) from mature leaves, whereas starch assimilation efficiencies are consistently lower (ca. 30-50%) (Horie *et al.*, 1985; Barbehenn *et al.*, 2014, 2015). Nevertheless, it is expected that overall CAE remains high on mature leaves, based on the predominance of sugars in the carbohydrate budgets of tree-feeding caterpillars (Horie *et al.*, 1985; Barbehenn *et al.*, 2014, 2015). The balance of protein and carbohydrate assimilated by insect herbivores can affect their growth rates (Clissold *et al.*, 2006; Behmer, 2009). Therefore, this study examines the effects of leaf maturation and wind stress on the balance of protein and carbohydrates that are assimilated by an herbivore.

Lymantria dispar is a highly polyphagous European species that was introduced into the eastern United States. It has become a model tree-feeding insect species because of its economic importance and wide host range. Among the hundreds of tree and shrub species on which *L. dispar* feeds, poplars are among the most favourable for its growth (Liebhold et al., 1995). Poplar has become a model deciduous tree because of its economic value, rapid growth, and the utility of *Populus* species for genetic research. In this study, rapid tree growth and large leaf size are essential for examining treatment effects in a brief (2 month) period with late-stadium larvae. Late-stadium larvae produce sufficient frass to quantify nutrient assimilation efficiencies for each individual. The use of a continuously growing (indeterminate) tree species such as poplar is

93 important to perform combined experiments on leaf maturity and wind effects because immature
94 and mature leaves are available together. The examination of wind stress in a greenhouse is
95 expected to increase the sensitivity of its detection, given that poplar trees are able to be grown in
96 the absence of wind for comparison with trees grown under wind stress.
97
98 The first main hypothesis tested is that leaf maturation has negative impacts on caterpillar
99 nutrition and growth due to decreased nutrient levels, decreased consumption rates, and
100 decreased nutrient assimilation efficiencies. To compare nutritional factors that could directly or
101 indirectly impact nutrient assimilation efficiencies and rates, levels of foliar protein,
102 carbohydrates, water, and fibre are measured. The second main hypothesis tested is that wind
103 stress has a negative impact on caterpillar nutrition and growth. A 2 x 2 factorial study is
104 performed on the immature and mature leaves from control and wind-blown poplar saplings,
105 examining leaf nutritional quality, nutrient assimilation efficiencies, and larval assimilation and
106 growth rates. Two factors that potentially affect nutrient assimilation efficiencies are also
107 examined: chewing efficiency (i.e., food particle size) and protein extraction efficiency from
108 ingested leaf tissues. If mature leaves are chewed less thoroughly than immature leaves, nutrient
109 assimilation efficiencies could be decreased. To determine whether ingested mature leaf tissues
110 retain higher levels of unextracted protein than do immature leaf tissues, protein levels are
111 measured in leaf particles from the midguts and frass of larvae that consume immature or mature
112 leaves. Together, these physiological measurements are aimed at a better understanding of the
113 impact of leaf quality on the nutritional ecology of forest Lepidoptera.
114
115

Materials and methods

Insect-plant system

Eggs of *L. dispar* were obtained from the USDA (Otis Air Force Base, Massachusetts). Larvae were reared in Petri dishes in incubators, primarily at 23 ^{o}C (16 h light). They were fed artificial diet (Addy, 1969) from egg hatch through the third or fourth stadium. The diet was modified by using linseed oil instead of wheat germ oil.

Poplar saplings of clone 717 (n = 20) were grown in a greenhouse in tall 8-L pots designed for tree growth (Barbehenn *et al.*, 2007). They were watered daily and fertilized with Osmocote 15-9-12 slow-release fertilizer (Hummert International, Earth City, Missouri) (50 mL per pot). Saplings attained a height of over 2 m before their leaves were used in the feeding experiment on leaf maturity (July, 2012). For the experiment on the effects of leaf maturation and wind stress (July, 2013), poplar saplings were grown as in the above experiment, with the exception that wind stress was applied to one group. Box fans were positioned to produce a wind speed averaging 2.1 m sec^{-1} on 9 saplings. The force of the air produced a constant swaying of the tree tops and movement of their leaves for more than a 2-month period before they were used. Saplings were moved to new positions daily. Wind speed was measured with an R.M. Young Wind Monitor-AQ anemometer (R.M. Young Co., Traverse City, Michigan). Control saplings (n = 9) were grown in the same room, but on the back side of the fans, where no measurable air speed was recorded. Average wind speeds outside the greenhouse were 1.3 m sec^{-1} (Barbehenn *et al*., 2015). Daily minimum and maximum temperatures in the greenhouse averaged 20.0 ± 0.6 and 29.7 ± 0.6 ^{o}C, respectively. All saplings produced over 40 leaves under the experimental conditions deployed.

Leaf position was determined using the leaf plastochron index (LPI), with the first uncurled leaf greater than 2 cm being defined as leaf "0", and older leaves counted radially around the tree (Larson & Isebrands, 1971). Very young leaves (less than LPI 10) were excluded from this study because they may contain elevated levels of toxins, feeding deterrents, and/or antinutritional compounds (e.g., salicylates and protease inhibitors), and are often avoided by foraging caterpillars (Meyer & Montgomery, 1983; Haruta *et al.*, 2001). For the first experiment, leaves were cut from LPI 10-12 (immature), LPI 20-22 (recently mature), and LPI 30-32 (fully mature). Immature leaves retained the same light green colour found in leaves from LPI 0-9, and they were not yet fully expanded. By comparison, mature leaves had become dark green. Leaves were cut with a razor blade through their petioles, and were harvested each morning during the experiments. This method was expected to avoid the induction of defences during the course of the experiment (Osier *et al.*, 2000). A single leaf was cut from each position from 9 trees each day. Leaf turgor was maintained by keeping the leaf petioles in tubes of water until they were used. Leaf discs (2.5 cm-diameter) were cut with a cork borer, avoiding the midrib. The discs were mixed within age groups to eliminate the potential effect of between-tree variation in food quality. The time from leaf harvest to larval feeding ranged between 2-3 hours. Leaf discs were kept turgid in a humidified Petri dish at ambient temperature before they were fed to larvae. The use of leaf disks permitted accurate measurements of consumption by providing amounts of food that would be largely consumed (Schmidt & Reese, 1986).

Immature leaves were selected for the wind stress experiment from a zone centred near LPI 15, and mature leaves were cut near LPI 25. Again, immature leaves were a light green colour and were not fully expanded. Leaf discs were obtained as described above, with the

exception that the leaves were first washed in a mild detergent solution, rinsed by soaking in tap water, and dried with paper towels before being cut into discs.

Effect of leaf maturity on larval nutrition and growth

Newly moulted fourth-stadium larvae were assigned randomly to one of three leaf age groups. Individual larvae were fed for a three-day period on immature, newly mature, or fully-mature leaves (n = 15 per treatment) in 35-mL snap-cap plastic cups. Groups of freshly cut leaf discs 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 ^{o}C) each day to determine the amount eaten. Representative leaf discs were weighed fresh and after drying to estimate the dry weight of food given to each larva on each day. Additional leaf discs were frozen (-80 ^{o}C) daily and lyophilized. Frass was collected daily, and kept separately for each larva in screw-cap centrifuge tubes at -80 ^{o}C. After 3 days of feeding, larvae were kept in empty cups for 6 h to collect their final frass pellets. Larvae were then frozen and dried to obtain their final masses. Initial larval masses were estimated from the fresh weight:dry weight ratio of 5 representative larvae. Consumption, assimilation and growth rates were relativized by dividing them by the initial dry mass of the larvae. Nutrient analyses are described below. The following nutritional indices were examined for protein:

Relative protein consumption rate (RPCR) = mass of protein ingested day^{-1} mg^{-1}

Protein assimilation efficiency (PAE) = (mass of protein ingested - mass of protein egested) / mass of protein ingested x 100

Relative protein assimilation rate (RPAR) = (mass of protein ingested - mass of protein egested) / day

(Note that RPCR x PAE = RPAR.) Protein assimilation efficiency is identical to the "approximate digestibility" of protein. Protein ingested was calculated as percent protein in leaves x total mass ingested, and total protein egested was calculated as percent protein in frass x total mass egested. Carbohydrate assimilation efficiencies and rates were measured in the same manner as described for protein by analyzing carbohydrate (i.e., sugar and starch) levels in the food and frass of the same larvae.

Effects of leaf maturity and wind stress on larval nutrition and growth

Newly moulted fifth-stadium larvae were weighed and assigned randomly to one of four treatment groups: immature control leaves, mature control leaves, immature wind-stressed leaves, or mature wind-stressed leaves. Larvae (n = 14 per treatment) were fed weighed leaf discs for a two-day period in the same experimental conditions as described above. At the end of the experiment, larvae were kept in empty cups overnight to collect their final frass pellets. A subgroup of larvae was dissected to confirm that their guts were empty after the period of starvation. Final larval weights were measured after they were dried (70 ^{o}C, 2 days). All leaf and frass samples were lyophilized for chemical analysis (below).

Factors affecting nutrient assimilation efficiency

Chewing. To examine whether chewing efficiency is impacted by leaf maturity, fifth-stadium larvae (n = 15per treatment) were fed for a 2-day period on leaf disks from immature, recently mature, or fully mature leaves grown in control conditions (July, 2012), as described above. Larvae were chilled individually (6 min, -20 ^{o}C) and dissected. The contents of the foreguts of

larvae were dispersed in 500 µL of 50% (v/v) methanol. Particles were stained and their sizes measured in micrographs as described previously (Barbehenn *et al.*, 2014).

Mandible size. To examine the effect of mandible size on food particle size, mandibles were dissected from the head capsules of each larva used for measuring food particle size. Mandible size was measured using an ocular micrometer in a stereo-microscope at a magnification of 25X, with the length between condyles (points of articulation) serving as an indicator of overall size.

Protein extractability from ingested leaf particles.

The ability of larvae to extract protein from ingested leaf tissues can decrease as leaves mature. To examine this directly, the protein that remained inside ingested leaf particles was measured in samples from the posterior half of the midguts of fifth-stadium larvae (July, 2012). These samples were obtained from the same larvae used for measuring food particle size. The contents of each posterior midgut (n = 15 per treatment) was placed in a separate tube containing 500 µL of 20 mM HCl, which was flushed with nitrogen. Samples were dispersed by shaking, and were immediately centrifuged (8,000 x ***g***, 3 min). Supernatant solutions were removed (eliminating any protein that was not contained inside the leaf particles), and the pellets were lyophilized. Protein-bound amino acids in 5-mg samples were measured in acid hydrolysates with high performance liquid chromatography (HPLC), as described below.

Protein levels were also compared in the frass of fifth-stadium larvae (n = 15 per treatment) in a separate experiment in which they fed on poplar leaves from one of the three age groups (July, 2012). The experiment followed the same procedures described above for feeding experiments with immature and mature leaves. Protein levels were measured in lyophilized frass

as total amino acids (protein-bound plus free) in acid hydrolysates with HPLC. Total amino acids in the frass were expected to be primarily protein-bound because previous work has found negligible levels of free amino acids in the frass of *L. dispar* larvae that fed on immature or mature red oak leaves (Barbehenn *et al*., 2014).

Chemical analyses

All analyses were performed as described previously (Barbehenn *et al.,* 2014). Briefly, protein was measured with HPLC as total amino acids (protein-bound plus free) in 6 M HCl hydrolysates from each leaf age and date and the frass from each larva. Of the total amino acids measured in the leaves, approximately 99% were expected to be protein-bound (Giovanelli, 1987; Ruuhola *et al.,* 2003). Sugars (summing glucose, fructose and sucrose) were measured using an enzymatic method (Zhao *et al.*, 2010). Starch was measured in the extracted pellets remaining from leaf and frass samples (Zhao *et al.*, 2010). Sugars and starch were combined as a measure of total nonstructural carbohydrates. Water was measured in each of the feeding experiments by weighing leaf discs before and after they were oven dried (70 ^{o}C). Total fibre was measured with the neutral detergent fibre (NDF) assay, modified from Van Soest & Wine (1967). In the experiment to examine the effects of wind stress, cellulose, hemicellulose and lignin were quantified as follows. The NDF assay was followed on the same samples by the acid detergent fibre (ADF) assay (Van Soest & Wine, 1967, as modified in Barbehenn *et al.,* 2014) and then an assay for lignin. Lignin (Klason type) was determined by treating the ADF pellets with 72% sulphuric acid (Dence, 1992). Cellulose was determined as the difference between ADF and lignin. Hemicellulose was determined as the difference between NDF and ADF.

Waste nitrogen products were quantified in the frass from each larva in the first experiment. The two major waste nitrogen products (uric acid and ammonium salts) were extracted from 5-mg frass samples in 500 µL of pH 2.1 buffer, and measured with HPLC as described previously (Barbehenn *et al.*, 2014). Allantoic acid and allantoin were below the limits of detection. Of the total amino acids (protein-bound + free) measured in the frass of *L. dispar*, approximately 98% were protein-bound, regardless of food quality (Barbehenn et al., 2014). Therefore, free amino acids are not considered a form of waste nitrogen for *L. dispar*.

Experimental design and statistical analyses

The experimental design in this study emphasizes the measurement of larval nutrition and growth to test the main hypotheses. The number of replicate larvae used (n = 14-15) was sufficient to demonstrate whether there are nutritional effects of leaf maturity and/or wind stress in a short period of larval growth (i.e., 2 or 3 days). By contrast, variation between trees was minimized by using a clone and mixing leaf samples from multiple saplings within treatment groups on each experimental day, leading to sample sizes of only n = 2 or 3 replicate days per treatment. This low number of replicates limited the statistical power to show treatment effects on the leaves. However, the experimental design was aimed primarily at quantifying the amounts of nutrients that were consumed by each larva.

The effects of leaf maturation on the levels of foliar protein, carbohydrate, water, fibre, were made between three leaf age groups with one-way ANOVA (SAS Institute, 2010). The effects of leaf maturation and wind stress on foliar nutritional quality were examined with a 2-factorial ANOVA, using leaf age and wind treatment as main factors. Because leaf discs were

pooled within treatment groups, replicates for leaf analyses were days within experiments (n = 2-3 per treatment).

The effects of leaf maturation on consumption rates and assimilation efficiencies were compared between leaf ages with ANCOVA, using ingested masses or egested masses as main factors and initial dry mass or ingested masses as covariates, respectively (SAS Institute, 2010). The assumption of parallel slopes for ANCOVA was confirmed with a separate analysis of the treatment x covariate interaction term (non-significant). Relative growth rate data could not be transformed to meet the assumptions of ANCOVA, and were examined with one-way ANOVA. The effects of leaf maturation and wind on larval nutrition and growth were compared with 2-factorial ANCOVA. One exception was CAE, which was analyzed with two-way ANOVA. Effects of leaf age on food particle protein, faecal protein, waste nitrogen, food particle size, and mandible size were examined between treatment groups with one-way ANOVA. For all analyses, 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, a *P*-value of 0.05 was used to determine statistical significance. Individual larvae served as replicates in all experiments on insects (see Methods and Results for sample sizes). Pearson correlation analyses were used to examine associations between (1) nutrient levels, consumption rates, assimilation efficiencies, assimilation rates, and growth rates, (2) mandible size and food particle size, and (3) P:C ratios in leaves and RPAR:RCAR by larvae.

Results

Effect of maturation on leaf nutritional quality

The levels of protein, carbohydrate, water and fibre were measured in the three ages of leaves ingested by larvae (Table 1). As often occurs in mature leaves, protein and water levels decreased significantly by 12-28% and 4-5%, respectively, relative to levels in immature leaves. However, changes in carbohydrate were not shown to be significant. Carbohydrate was composed of 87-92% sugar, with the remaining fraction being starch. Fibre levels were significantly higher by 20% in fully mature leaves.

Effect of leaf maturation on larval nutrition and growth

Consistent with the first hypothesis, larval consumption rates were 29-32% higher on immature leaves than on mature leaves (Table 2). Although assimilation efficiencies for the entire leaf mass decreased when larvae fed on mature leaves, the assimilation efficiencies of two major macronutrients were not impacted negatively by leaf maturation. Protein assimilation efficiency remained high (74-82%) in *L. dispar* caterpillars feeding on mature leaves. Carbohydrate assimilation efficiency also remained high in larvae on all ages of poplar leaves (Table 3). Sugars were assimilated with exceptionally high efficiencies (averaging 94-97% across leaf ages), whereas starch was assimilated less efficiently (averaging 56-60%). However, the relatively small amounts of starch in poplar leaves meant that low starch assimilation efficiency had little effect on overall CAE. Waste nitrogen produced by larvae on mature leaves dropped by 43-60% (Table 4). The total amount of waste nitrogen produced by each larva was associated positively with the total amount of protein it assimilated ($r = 0.74$, $P < 0.001$). The growth rates of larvae that fed on mature leaves declined by 33-43%, in association with lower

consumption rates ($r = 0.74$), lower RPAR ($r = 0.86$), and to a lesser extent, lower RCAR ($r = 0.41$).

Effect of leaf maturation and wind stress on leaf nutritional quality

During leaf maturation, protein levels were again found to decrease significantly by 24%, whereas the opposite trend in carbohydrates was observed. In contrast to the first experiment, no significant changes in leaf water content or trends in fibre levels were observed in mature leaves. Wind-stressed leaves contained fibre levels that were increased significantly by 3-5% relative to the levels in control trees (Table 5). Among the three fibre components measured, wind stress was associated with a trend towards higher cellulose ($P = 0.112$), but no increases in lignin or hemicellulose were detected (Fig. 1).

Effect of leaf maturation and wind stress on larval nutrition and growth

Consistent with the results of the above experiment on leaf maturation, the PAE and CAE of larvae that fed on mature poplar leaves remained high (ca. 70% and 90%, respectively) (Tables 6 and 7). The negative effect of leaf maturation on PAE was small (5-9% decrease), as was the positive effect of leaf maturation on CAE (3-4% increase). Contrary to the wind stress hypothesis, *L. dispar* larvae that fed on wind-stressed leaves did not have significantly decreased PAE or CAE. However, there was a significant 4-7% decrease in total assimilation efficiency in larvae on wind-stressed leaves, which could not be explained by patterns of protein or carbohydrate assimilation. In addition, larvae that fed on mature wind-stressed leaves had significantly lower consumption rates, which led to a trend towards lower growth rates on wind-stressed leaves (Table 6). The importance of protein nutrition for larval growth can be inferred

345 from correlations between nutrient assimilation rates and growth rates. Larval growth rates
346 (Table 6) were closely associated with protein assimilation rates (RPAR; $r = 0.74$), and more
347 strongly associated with protein than with carbohydrate assimilation rates (RCAR; $r = 0.53$). In
348 turn, RPAR and RCAR were strongly associated with protein and carbohydrate consumption
349 rates ($r = 0.99$ and 0.98, respectively). Protein assimilation efficiency, which contributes to
350 RPAR, was also associated with RPAR ($r = 0.74$).
351
352 Consistent with the efficient assimilation of protein and carbohydrate from immature and
353 mature poplar leaves, foliar protein : carbohydrate (P:C) ratios were strongly associated with
354 larval RPAR : RCAR ratios (Fig. 2, $r = 0.97$, $P < 0.001$ for combined data). The slope of the
355 regression equation was close to 1.0 (0.88), indicating that the ratio of P:C assimilated was
356 modified slightly from the P:C ratio in the leaves.
357
358 *Factors affecting nutrient assimilation efficiency*
359 *Chewing*. Caterpillars chewed immature and mature leaves into a wide range of particle sizes
360 (Fig. 3). Contrary to expectation, median food particle sizes were similar between each leaf age
361 group, ranging from 0.32-0.34 μm^2. Larval mandibles ranged from 0.89 to 1.22 mm wide.
362 However, mandible size was not associated with food particle size ($r = 0.15$-0.34 for each leaf
363 age).
364
365 *Protein extractability from ingested leaf particles*. Protein levels in food particles from the
366 posterior midgut did not differ significantly between larvae on immature and mature leaves (Fig.
367 4), consistent with the high PAEs in *L. dispar* on all ages of poplar leaves. Protein levels

368 remaining in the frass of larvae that fed on immature or mature leaves also supports the
369 conclusion that protein does not become more difficult to extract from mature leaves (Fig. 4).
370 The large difference in protein levels between the posterior half of the midgut and the frass
371 suggests that much of the absorption of amino acids occurs in the posterior midgut.
372
373 **Discussion**
374 The larvae of *L. dispar* grow more rapidly on immature than on mature poplar leaves, as
375 would be expected from previous research (Feeny, 1970; Hough & Pimentel, 1978; Schweitzer,
376 1979; Raupp & Denno, 1983; Raupp et al., 1988; Schroeder, 1986; Hunter & Lechowicz, 1992;
377 Parry *et al*., 1998; Kursar & Coley, 2003). However, by examining a wide range of
378 physiological factors that potentially impact herbivore growth, this study shows that their higher
379 growth rates on immature poplar leaves are due to higher leaf quality, higher consumption rates,
380 and the associated increases in nutrient assimilation rates, but do not result from higher nutrient
381 assimilation efficiencies or greater chewing efficiency by larvae on lush young leaves. Thus, the
382 findings in this study require the rejection of one component of the first hypothesis: PAE and
383 CAE by *L. dispar* larvae are not negatively impacted when larvae feed on mature poplar leaves.
384 Similar, high PAE (77-80%) and CAE (83-87%) are also observed in fifth-stadium larvae on
385 immature or mature poplar leaves (R. V. Barbehenn, unpublished observations). This is contrary
386 to a common belief that nutrient assimilation efficiencies decrease in insect herbivores on mature
387 or more fibrous leaves (e.g., Read & Stokes, 2006). Whereas there is a pattern of decreased PAE
388 from the mature leaves of some trees, such as red oak and sugar maple (Barbehenn *et al.*, 2013a,
389 2014), there is a large amount of variation in this trait among tree species. Several species in the
390 Salicaceae have highly available protein and carbohydrates in their mature leaves (Barbehenn *et*

391 *al.*, 2015). The present study confirms this finding, and offers a more detailed view of the
392 nutritional physiology of *L. dispar* on a wider range of leaf ages.
393
394 A second major finding is that wind-stress during poplar leaf development has only a
395 limited impact on foliar composition and herbivore nutrition. Wind-stressed leaves develop
396 higher levels of fibre, presumably as an adaptive response to avoid mechanical damage. In one
397 of the few previous studies on wind stress that examine foliar chemical composition, wind stress
398 causes bean leaves to produce higher levels of fibre, especially lignin (Cipollini, 1997).
399 However, lignin is not among the fibre components that increase in wind-stressed poplar. Also
400 contrary to the wind stress hypothesis, the changes in wind-stressed poplar leaves do not
401 significantly affect the availability of nutrients to *L. dispar* larvae. The results of this study
402 confirm recent results showing that when poplar is grown in outdoor wind conditions, protein
403 and carbohydrate are assimilated efficiently from mature leaves (i.e., PAE = 74-75% and CAE =
404 78%; Barbehenn *et al.*, 2015). In the only previous study on the effects of wind stressed leaves
405 on a leaf-chewing herbivore, the growth rate of *M. sexta* caterpillars is not affected significantly
406 by feeding on wind-stressed tomato leaves (Cipollini & Redman, 1999). Similarly, in the current
407 study, there are only limited effects of wind stressed leaves on the nutrition of *L. dispar*, and
408 these appear to be attributable to decreased consumption rate rather than decreased nutrient
409 availability.
410
411 To understand the adverse effects of feeding on mature leaves, several nutritional
412 mechanisms are examined in this study, including chewing efficiency, protein extractability, P:C
413 ratios, and waste nitrogen excretion. The ability of *L. dispar* to chew leaves into fine particles is

414 the same on immature and mature poplar leaves. This is unexpected, given that cell wall
415 thickness increases on the order of ten-fold and fibres become extensively crosslinked during
416 maturation (Brisson *et al.*, 1994; Doblin *et al.*, 2003). However, similar chewing abilities are
417 observed in larvae on immature and mature red oak leaves, the latter of which are remarkably
418 tough (Barbehenn *et al.*, 2013a, 2014). Therefore, in each of these studies on *L. dispar*,
419 increased fibre levels and toughness do not affect nutrient assimilation efficiencies as a result of
420 chewing efficiency. In caterpillars on oak, PAE decreases markedly on mature leaves, whereas
421 in caterpillars on poplar, PAE remains high, regardless of the similar sizes of leaf particles that
422 are chewed by larvae on the immature or mature leaves of each host plant.
423
424 Although leaf toughness (or fibre content) does not affect the chewing efficiency of *L.*
425 *dispar* with respect to food particle size, it does appear to impact the rate of food consumption
426 for *L. dispar* and many other insect herbivores (Choong, 1996; Kursar & Coley, 2003;
427 Barbehenn *et al.*, 2013, 2014, 2015). For *L. dispar*, there are strong associations between
428 consumption rate, protein assimilation rate, and growth rate. Importantly, decreased growth rate
429 commonly leads to decreased fitness (Scriber & Slansky, 1981; Awmack & Leather, 2002).
430
431 Consumption rates could also be decreased on mature leaves by decreased levels of
432 feeding stimulants and/or increased levels of deterrent or toxic plant compounds. It is
433 noteworthy that sugars (feeding stimulants) remain at equally high levels in mature and
434 immature poplar leaves. The major deterrents and toxins in *Populus* species are salicylates (or
435 "phenolic glycosides"), but these are found at lower levels in mature than immature leaves (Osier
436 *et al.*, 2000; Kleiner *et al.*, 2003), presumably causing *L. dispar* larvae to avoid feeding on the

437 youngest leaves (Meyer & Montgomery, 1987). Previous work shows that the poplar leaves
438 used in this study have negligible levels of tannins and low levels of other phenolic compounds
439 (Barbehenn *et al.,* 2007). It is also unlikely that some of the putative defences examined
440 previously in wind stressed leaves, e.g., peroxidase and polyphenol oxidase, affect the nutrition
441 of *L. dispar* (Barbehenn *et al.,* 2007, 2010, 2012). Further work would be needed to examine
442 whether other types of feeding stimulants and/or deterrents affect the consumption rates by *L.*
443 *dispar* on mature poplar leaves (e.g., Coyle *et al.,* 2002).
444
445 Protein extractability is a key difference between the digestibility of the leaf tissues of
446 poplar and the leaf tissues of species such as red oak. Whereas protein remains highly
447 extractable from the ingested tissues of mature poplar leaves, protein in mature red oak leaves
448 becomes significantly more difficult to extract (Barbehenn *et al.,* 2014). In contrast to protein,
449 CAE remains equally high in *L. dispar* larvae that feed on the immature or mature leaves of
450 poplar or red oak. Therefore, the ratios of protein : carbohydrate present in immature and mature
451 poplar leaves are tightly associated with the ratios of RPAR:RCAR in *L. dispar* larvae. Similar
452 results are found in *L. dispar* on a several species of trees in the Salicaceae (Barbehenn *et al.,*
453 2015). These findings reiterate the minimal effect that leaf maturation has on macronutrient
454 extractability from certain host plants, in comparison to the large decrease in PAE from species
455 such as red oak and sugar maple (Barbehenn *et al.,* 2013, 2014). Shifts in the balance of protein
456 and carbohydrates that can be assimilated from mature leaves could be important for the growth
457 of *L. dispar*, as found in other insect herbivores on other food sources (Clissold *et al.,* 2006;
458 Behmer, 2009). Together, these studies of *L. dispar* on a variety of tree species show that

459 developmental changes in leaf tissues can have a strong effect on nutrient availability, but that
460 this effect varies greatly between tree species and nutrient types.
461
462 Given that larvae of *L. dispar* appear to maximize protein and carbohydrate assimilation
463 efficiencies, it is likely that post-absorptive mechanisms are important to deal with unneeded
464 nutrients (e.g., Zanotto *et al.*, 1993; Trier & Mattson, 2003). This study examines waste nitrogen
465 production, but not the metabolism of carbohydrates. Waste nitrogen production by *L. dispar* is
466 proportional to the amount of protein metabolized, as found previously in larvae feeding on red
467 oak (Barbehenn *et al.*, 2014). This could either indicate that protein is in excess supply in
468 immature leaves or that waste nitrogen products must be excreted in larger amounts as larvae
469 metabolize more protein. The strong association between protein assimilation rates and growth
470 rates in larvae feeding on all leaf ages suggests that they effectively utilized the protein for
471 growth at all protein levels found in poplar and oak, rather than facing an excess of protein in
472 immature leaves. Instead, the production of waste nitrogen may largely result from the mismatch
473 between the amino acid profiles of foliar and larval protein (Barbehenn *et al.*, 2013b), i.e.,
474 assimilated amino acids that do not match the pattern required for insect protein synthesis must
475 be metabolized, resulting in the excretion of waste nitrogen products in proportion to ingested
476 protein.
477
478 In conclusion, the results of the current study show that protein and carbohydrate can be
479 assimilated efficiently by *L. dispar* caterpillars feeding on the leaves of poplar trees, regardless
480 of leaf maturity. Leaf development under wind stress does not significantly alter these findings.
481 Instead, the negative impacts of feeding on older (basal) leaves are related to the decreased levels

of certain nutrients, such as protein, and to decreased consumption rates. Together, these factors limit nutrient assimilation rates, and ultimately growth. Further work is needed to examine the potential importance of leaf toughness and compounds that act as antifeedants or toxins in mature leaves. Finally, the mechanisms that limit protein extractability from the mature leaves of some tree species are unknown. It is speculated that the chemical and structural properties of cell walls may explain the variation between the extractability of protein from leaf tissues, but the authors are unaware of any work that bears on this topic. Additional studies are needed to compare nutrient availabilities from the immature and mature leaves of a wider range of tree species to determine whether species in the Salicaceae are unusual, or whether there are general patterns across taxa, such as between indeterminate and determinate tree species. These studies will provide a better understanding of the physiological mechanisms underlying the variation in host plant quality for insects such as *L. dispar*.

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Figure legends

Fig. 1. Fibre composition of immature and mature poplar leaves (*Populus alba* x *P. tremula*) grown without or with wind stress. Fibre components are expressed as a percentage of the total dry weight of the leaf, and sum to equal total fibre (NDF). DW = dry weight.

Fig. 2. Association between protein : carbohydrate ratios in immature and mature poplar leaves and the ratios of protein : carbohydrate assimilation rates (PAR:CAR or RPAR:RCAR) in larvae of the gypsy moth *Lymantria dispar*. ●, leaf age experiment; ◆, wind-stress experiment; ▲, R.V. Barbehenn, unpublished observations. $r = 0.97$, $P < 0.001$.

Fig. 3. Particle size distributions in the foreguts of *L. dispar* larvae after feeding on immature, recently mature, and mature poplar leaves.

Fig. 4. Protein levels remaining in rinsed food particles from the posterior half of the midgut and in the frass from *Lymantria dispar* larvae after feeding on immature, recently mature, and mature poplar leaves (separate experiments). Bars show mean ± standard error, and letters above bars that differ within each series designate $P < 0.05$.

Table 1. Nutritional quality of immature and mature poplar leaves (*Populus alba* x *P. tremula*)[1]

Leaf age	Protein (% DW)	Carbohydrate[2] (% DW)	Water (% FW)	Fibre (% DW)
Immature	24.2 ± 0.7 [b]	8.80 ± 1.41	76.0 ± 0.3[b]	26.1 ± 1.4 [a]
Recently mature	21.4 ± 1.4 [a]	10.52 ± 0.17	72.6 ± 0.7[a]	26.1 ± 1.8 [a]
Mature	17.3 ± 0.7 [a]	9.39 ± 0.73	72.1 ± 0.7[a]	31.2 ± 0.6 [b]
	P = 0.010	P = 0.203	P = 0.006	P = 0.017

[1]Leaf ages represent LPI numbers 10-12, 18-20, and 29-30, respectively. Data are presented as mean ± SE, with n = 3 replicate days/leaf age for all measurements. Summary statistics followed by different letters are significantly different (P < 0.05).

[2]Carbohydrate includes sugars (87-92% of total) and starch.

Table 2. Consumption and assimilation of protein from immature and mature hybrid poplar leaves (*Populus alba* x *P. tremula*) and growth rates of *Lymantria dispar* larvae feeding on them.

Leaf age	Relative consumption rate (mg mg^{-1} day^{-1})	Assimilation efficiency (%)	Relative protein consumption rate (mg mg^{-1} day^{-1})	Protein assimilation efficiency (%)	Relative protein assimilation rate (mg mg^{-1} day^{-1})	Relative growth rate (mg mg^{-1} day^{-1})
Immature	3.14 ± 0.12 [b]	40.5 ± 0.6 [b]	0.755 ± 0.043 [c]	81.5 ± 0.8 [a]	0.614 ± 0.036 [c]	0.870 ± 0.054 [b]
Recently mature	2.43 ± 0.10 [a]	34.9±1.2 [a]	0.517 ± 0.007 [b]	78.2 ± 0.7 [a]	0.404 ± 0.024 [b]	0.586 ± 0.030 [a]
Mature	2.37 ± 0.09 [a]	35.5 ± 0.9 [a]	0.408 ± 0.021 [a]	74.4 ± 1.1 [a]	0.304 ± 0.015 [a]	0.497 ± 0.027 [a]
	$P < 0.001$	$P = 0.002$	$P < 0.001$	$P = 0.187$	$P < 0.001$	$P < 0.001$

Leaf ages represent LPI numbers 10-12, 18-20, and 29-30, respectively. Data are presented as mean ± SE, n = 13-15 for all measurements. Assimilation efficiency = (mass ingested – mass egested) / mass ingested * 100.

Table. 3 Consumption and assimilation of carbohydrates from immature or mature hybrid poplar leaves by *Lymantria dispar* larvae

Leaf age	Relative carbohydrate consumption rate (mg mg^{-1} day^{-1})	Carbohydrate assimilation efficiency (%)	Relative carbohydrate assimilation rate (mg mg^{-1} day^{-1})
Immature	0.288 ± 0.018^{b}	83.8 ± 0.5^{a}	0.241 ± 0.016^{ab}
Recently mature	0.268 ± 0.012^{b}	87.1 ± 0.8^{b}	0.234 ± 0.011^{b}
Mature	0.223 ± 0.011^{a}	87.2 ± 0.7^{b}	0.195 ± 0.009^{a}
	$P = 0.032$	$P = 0.004$	$P = 0.073$

Leaf ages represent LPI numbers 10-12, 18-20, and 29-30, respectively. Summary statistics followed by different letters are significantly different ($P < 0.05$). Assimilation efficiency = (mass ingested – mass egested) / mass ingested * 100.

Table 4. Waste nitrogen excretion by *Lymantria dispar* larvae that fed on immature or mature poplar leaves

Leaf age	Uric acid (% DW)	Ammonium salts (% DW)	Total waste nitrogen (%DW)
Immature	0.228 ± 0.017 [a]	1.03 ± 0.11 [b]	1.257 ± 0.116 [b]
Recently mature	0.196 ± 0.017 [a]	0.75 ± 0.08 [a]	0.959 ± 0.097 [a]
Mature	0.219 ± 0.023 [a]	0.60 ± 0.09 [a]	0.879 ± 0.084 [a]
	P = 0.529	P = 0.012	P = 0.034

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

Table 5. Nutritional quality of immature and mature poplar leaves grown without or with wind stress

Treatment	Leaf age	Protein (%DW)	Carbohydrate (%DW)	Water (% FW)	Fibre (%DW)
Control	Immature	26.1 ± 0.6 [b]	7.2 ± 0.7 [ab]	69.7 ± 0.7 [a]	24.4 ± 0.3 [ab]
Control	Mature	19.7 ± 1.1 [a]	8.2 ± 0.5 [b]	71.1 ± 1.5 [a]	24.2 ± 0.1 [a]
Wind	Immature	26.3 ± 0.8 [b]	5.8 ± 0.2 [a]	69.7 ± 0.6 [a]	25.1 ± 0.3 [ab]
Wind	Mature	19.9 ± 0.2 [a]	7.3 ± 0.6 [ab]	71.7 ± 1.6 [a]	25.4 ± 0.4 [b]
Treatment		$P = 0.757$	$P = 0.098$	$P = 0.845$	$P = 0.031$
Leaf age		$P = 0.001$	$P = 0.079$	$P = 0.370$	$P = 0.783$
Treatment x leaf age		$P = 0.993$	$P = 0.693$	$P = 0.862$	$P = 0.426$

Leaf ages represent LPI numbers near 15 and 25, respectively. Data are presented as mean ± SE, with only $n = 2$ replicate dates/treatment and leaf age. Summary statistics followed by different letters are significantly different ($P < 0.05$).

Table 6. Consumption and assimilation of protein and growth of *Lymantria dispar* larvae on immature or mature leaves of poplar grown without or with wind stress

Treatment	Leaf age	Relative consumption rate (mg mg^{-1} day^{-1})	Total assimilation efficiency (%)	Relative protein consumption rate (mg mg^{-1} day^{-1})	Protein assimilation efficiency (%)	Relative protein assimilation rate (mg mg^{-1} day^{-1})	Relative growth rate (mg mg^{-1} day^{-1})
Control	Immature	1.21 ± 0.04 ^{b}	36.6 ± 0.7 ^{b}	0.315 ± 0.010 ^{c}	76.1 ± 0.5 ^{c}	0.245 ± 0.010 ^{c}	0.189 ± 0.011 ^{b}
Control	Mature	1.18 ± 0.06 ^{b}	40.2 ± 0.8 ^{c}	0.232 ± 0.012 ^{b}	72.1 ± 1.3 ^{ab}	0.161 ± 0.006 ^{b}	0.179 ± 0.013 ^{b}
Wind	Immature	1.11 ± 0.04 ^{b}	34.2 ± 0.7 ^{a}	0.291 ± 0.010 ^{c}	75.4 ± 0.8 ^{bc}	0.234 ± 0.009 ^{c}	0.181 ± 0.009 ^{b}
Wind	Mature	0.86 ± 0.03 ^{a}	38.4 ± 1.0 ^{ab}	0.172 ± 0.007 ^{a}	68.8 ± 0.9 ^{a}	0.117 ± 0.004 ^{a}	0.140 ± 0.006 ^{a}
Treatment		$P < 0.001$	$P = 0.008$	$P < 0.001$	$P = 0.155$	$P = 0.003$	$P = 0.080$
Leaf age		$P < 0.001$	$P < 0.001$	$P < 0.001$	$P = 0.004$	$P < 0.001$	$P = 0.007$
Treatment x leaf age		$P = 0.008$	$P = 0.979$	$P = 0.032$	$P = 0.790$	$P = 0.007$	$P = 0.099$

Leaf ages represent LPI numbers near 15 and 25, respectively. Data are presented as mean ± SE, n = 10-14 for all measurements. Summary statistics followed by different letters are significantly different ($P < 0.05$). Assimilation = (mass ingested – mass egested).

Table 7. Consumption and assimilation of carbohydrates by *Lymantria dispar* larvae on immature or mature leaves of poplar grown without or with wind stress

Treatment	Leaf age	Relative carbohydrate consumption rate (mg mg^{-1} day^{-1})	Carbohydrate assimilation efficiency (%)	Relative carbohydrate assimilation rate (mg mg^{-1} day^{-1})
Control	Immature	0.085 ± 0.003 [b]	90.0 ± 0.6 [a]	0.076 ± 0.003 [b]
Control	Mature	0.094 ± 0.004 [c]	92.8 ± 0.3 [b]	0.087 ± 0.004 [c]
Wind	Immature	0.061 ± 0.002 [a]	88.7 ± 0.6 [a]	0.054 ± 0.002 [a]
Wind	Mature	0.061 ± 0.003 [a]	92.7 ± 0.3 [b]	0.057 ± 0.003 [a]
Treatment		$P < 0.001$	$P = 0.143$	$P < 0.001$
Leaf age		$P = 0.073$	$P < 0.001$	$P = 0.005$
Treatment x leaf age		$P = 0.119$	$P = 0.210$	$P = 0.120$

Leaf ages represent LPI numbers near 15 and 25, respectively. Data are presented as mean ± SE, n = 10-14 for all measurements except carbohydrate assimilation efficiency and P:C assimilation ratios (n = 9-10). Summary statistics followed by different letters are significantly different ($P < 0.05$). Assimilation = (mass ingested – mass egested).

Fig. 1

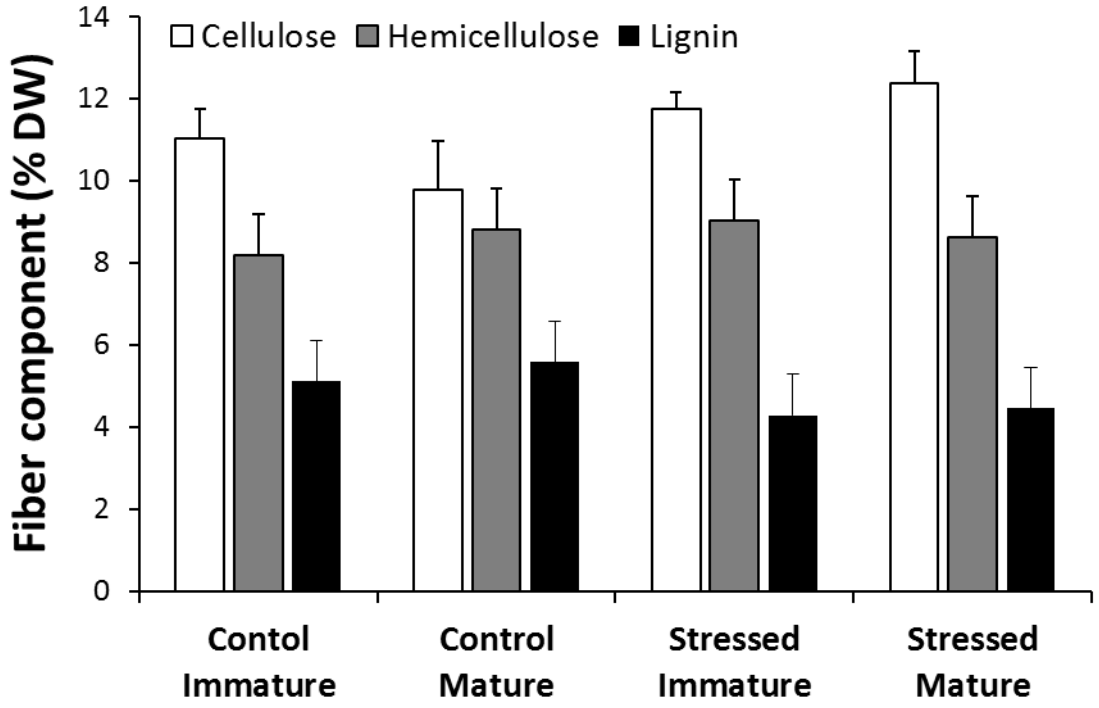

Fig. 2

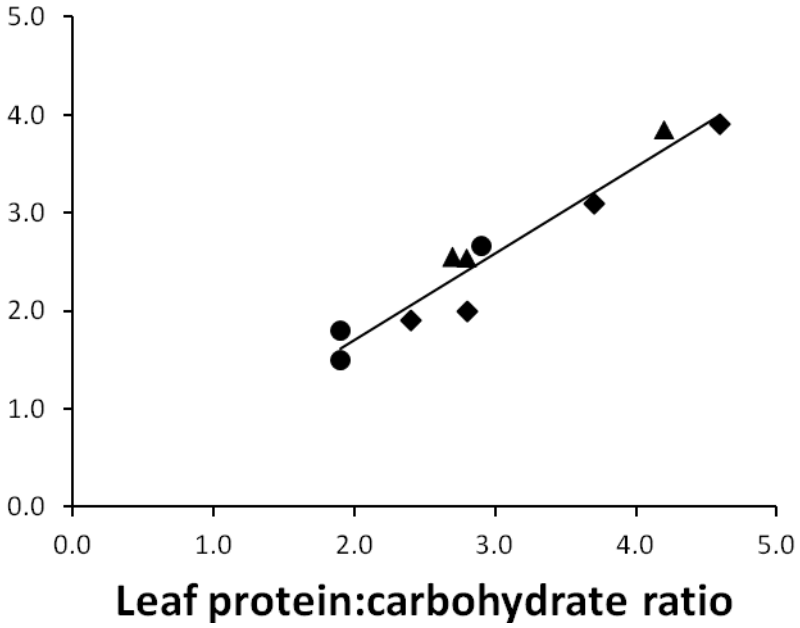

Fig. 3

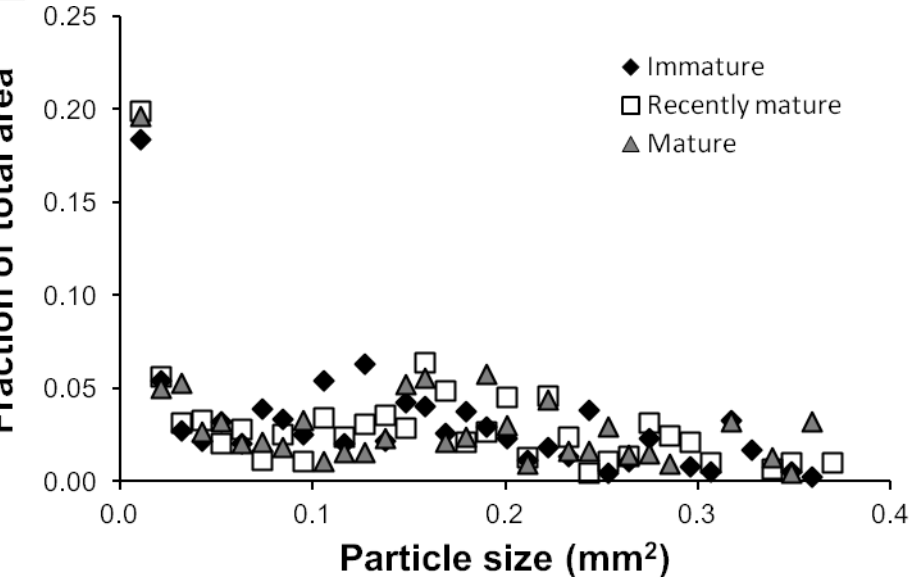

Fig. 4