Physiological Entomology

Physiological Entomology (2015) **40**, 72–81 DOI: 10.1111/phen.12087

Nutrients are assimilated efficiently by *Lymantria dispar* caterpillars from the mature leaves of trees in the Salicaceae

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Abstract. The efficient aquisition of nutrients from leaves by insect herbivores increases their nutrient assimilation rates and overall fitness. Caterpillars of the gypsy moth (*Lymantria dispar* L.) have high protein assimilation efficiencies (PAE) from the immature leaves of trees such as red oak (*Quercus rubra*) and sugar maple (*Acer saccharum)* (71–81%) but significantly lower PAE from their mature leaves (45–52%). By contrast to this pattern, both PAE and carbohydrate assimilation efficiencies (CAE) remain high for *L. dispar* larvae on the mature leaves of poplar (*Populus alba* × *Populus tremula*) grown in greenhouse conditions. The present study tests two alternative hypotheses: (i) outdoor environmental stresses cause decreased nutrient assimilation efficiencies from mature poplar leaves and (ii) nutrients in the mature leaves of trees in the poplar family (Salicaceae) remain readily available for *L. dispar* larvae. When poplar trees are grown in ambient outdoor conditions, PAE and CAE remain high (approximately 75% and 78%, respectively) in *L. dispar* larvae, in contrast to the first hypothesis. When larvae feed on the mature leaves of species in the Salicaceae [aspen (*Populus tremuloides*), cottonwood (*Populus deltoides*), willow (*Salix nigra*) and poplar], PAE and CAE also remain high (68–76% and 72–92%, respectively), consistent with the second hypothesis. Larval growth rates are strongly associated with protein assimilation rates, and more strongly associated with protein assimilation rates than with carbohydrate assimilation rates. It is concluded that tree species in the Salicaceae are relatively high-quality host plants for *L. dispar* larvae, in part, because nutrients in their mature leaves remain readily available.

Key words. Assimilation, carbohydrates, digestion, foliar nutrients, herbivore, host plant, larval growth, Lepidoptera, protein.

Introduction

The fitness of insect herbivores, including parameters such as growth rate, final weight and fecundity, is greatly affected by plant nutritional quality (Scriber & Slansky, 1981; Awmack & Leather, 2002). Nutritional quality is determined by a mix of factors, including levels of protein, carbohydrates, water and fibre, as well as the ratio of protein to carbohydrates. These factors vary greatly between tree species, environmental conditions and leaf ages (Rickleffs & Matthew, 1982; Raupp & Denno, 1983). Among the macronutrients, protein is often considered the most limiting for the performance of a wide range of herbivores (Feeny, 1970; Mattson, 1980; Coley *et al*., 2006; Wallis *et al*., 2012).

Protein availability is a function of the level of protein in leaves, as well as the ability of herbivores to extract, digest and absorb it. The level of protein declines rapidly in deciduous

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tree leaves during their early expansion, after which levels frequently remain relatively stable (Feeny, 1970; Schroeder & Malmer, 1980; Wint, 1981; Aide & Londono, 1989; Williams *et al*., 1998; Kursar & Coley, 2003; Barbehenn *et al*., 2013). Few studies have compared protein assimilation efficiency (PAE) from immature and mature leaves by insect herbivores (Schroeder, 1986). This measure combines the effects of protein extraction, digestion and absorption efficiencies. The maturation of red oak and sugar maple (*Acer saccharum*; Aceraceae) leaves significantly decreases protein extractability and/or PAE in *Lymantria dispar* L. larvae, with PAE falling from 71–81% to 45–52% (Barbehenn *et al*., 2013, 2014). Once extracted, protein digestion and amino acid absorption appear to be rapid processes (Martin *et al*., 1987; Woods & Kingsolver, 1999; but see also Clissold *et al*., 2010). Potential inhibitors of protein digestion in plants, such as tannins and protease inhibitors, do not explain the decrease in PAE in insect herbivores on mature leaves. By contrast to early studies on tannins, insect herbivores have a variety of mechanisms for avoiding the antidigestive effects of these compounds (Barbehenn & Constabel, 2011). Moreover, protease inhibitors have been found at higher levels in immature than mature leaves, which is the opposite of the pattern expected if they decreased PAE from mature leaves (Cipollini & Bergelson, 2000; Haruta *et al*., 2001).

The availability of carbohydrates is affected by foliar carbohydrate levels, as well as by the types of carbohydrates present. Whereas sugars are assimilated with high efficiencies, often exceeding 90% (Horie *et al*., 1985; Barbehenn *et al*., 2014), starch assimilation efficiencies in caterpillars are typically much lower (e.g. 20–60%) (Waldbauer, 1968; Harvey, 1975; Barbehenn *et al*., 2014). In the present study, it is expected that carbohydrate assimilation efficiency (CAE) is strongly affected by carbohydrate composition. Thus, carbohydrate composition may also affect the balance of protein to carbohydrate (P : C ratio) that is assimilated by insect herbivores. Although it is well established that P : C ratios in artificial diets play an important role in insect performance (Behmer, 2009), there are few studies relating the P : C ratios in leaves to the P : C ratios assimilated by herbivores.

Based on the decreased PAE of *L. dispar* larvae on the mature leaves of red oak and sugar maple, it is expected that this is a common phenomenon. Thus, the recent finding that the PAE of *L. dispar* on poplar (*Populus alba* × *Populus tremula*) remains high on mature leaves (i.e. PAE = 72–82%) is unexpected (R. V. Barbehenn, N. Haugberg, J. Kochmanski & B. Menachem, unpublished observations). The poplar leaves used in the unpublished study grew in a greenhouse, and it is possible that low levels of environmental stress affected their nutritional quality; for example, by the production of low levels of lignin (Cipollini, 1997; Kaur *et al*., 2012). A recent test of the hypothesis that wind-stressed poplar leaves have decreased nutrient availability shows that PAE and CAE remain high (approximately 70% and 90%, respectively) for *L. dispar* larvae on mature poplar leaves, whether the trees are grown in windy conditions or still air (R. V. Barbehenn, N. Haugberg, J. Kochmanski & B. Menachem, unpublished observations). However, attenuated sunlight levels (especially ultraviolet light) also affect the physical and chemical properties of poplar leaves (Warren *et al*., 2002; Mellway *et al*., 2009). Therefore, a major goal of the present study is to examine the impact of ambient outdoor conditions on the nutritional quality of mature poplar leaves.

The caterpillar species used in the present study, *L. dispar*, is a broad generalist, feeding on approximately 200 species of trees and shrubs in eastern North America (Liebhold *et al*., 1995). Species in the Salicaceae are among the most favourable host plants for larval growth (Liebhold *et al*., 1995). Although *L. dispar* larvae begin feeding in the early spring on immature leaves, they feed extensively on mature leaves in the late spring to complete their development (Hamilton & Lechowicz, 1991). Previous work shows that the growth rates and survivorship of *L. dispar* are significantly decreased in less than 1 week during leaf maturation in some tree species (Raupp *et al*., 1988). Therefore, an examination of the effects of leaf maturation on nutrient availability and larval growth performance is important for understanding the fitness of spring-feeding Macrolepidoptera such as *L. dispar*.

The present study tests two alternative hypotheses: (i) outdoor environmental stresses cause decreased nutrient availability from mature poplar leaves and (ii) foliar nutrients in trees in the poplar family (Salicaceae) remain readily available after their leaves mature. Rather than examine the specific effects of light on nutrient availability in the present study, the overall effects of poplar leaf maturation in ambient sun and wind conditions on PAE and CAE are tested. For the purposes of the present study, it is unnecessary to investigate individual abiotic stresses if the combination of all stresses does not lead to decreased nutrient availability. To test whether the high availability of nutrients from mature poplar leaves is typical of other species in the Salicaceae, PAE and CAE are measured in *L. dispar* on trembling aspen (*Populus tremuloides*), eastern cottonwood (*Populus deltoides*) and black willow (*Salix nigra*), as well as poplar. Support for the second hypothesis would be found if PAE and CAE are high in *L. dispar* on the mature leaves of poplar and the other species of Salicaceae. A range of foliar nutritional factors that potentially affect larval performance are measured, including protein, carbohydrates, water and fibre. In addition to assimilation efficiencies, the assimilation rates of nutrients are also determined because these are expected to be good predictors of larval growth rates. Finally, associations between P : C ratios in leaves and the ratios of P : C assimilation rates by *L. dispar* larvae are examined to determine whether larvae modify the levels of nutrients that they assimilate to obtain a specific P : C ratio.

Materials and methods

Insects

Eggs of *L. dispar* were obtained from the United States Department of Agriculture (Otis Air Force Base, Massachusetts). Larvae were reared in an incubator, primarily at 23 °C (16 h light) on an artificial diet (Addy, 1969). The diet was modified by using linseed oil instead of wheat germ oil. Larvae were reared in groups in Petri dishes, and were fed fresh diet daily until they reached the fourth stadium.

Trees

Poplar saplings ($n = 6$) were clones of a *P. alba* × *P. tremula* hybrid (717). They were grown initially in a greenhouse in 8-L pots, as described previously (Barbehenn *et al*., 2007). Mid-afternoon temperatures in fair weather in the greenhouse were (mean ± SE) 29.7 ± 0.6 °C. Photosynthetically active radiation (PAR) and ultraviolet (UV) radiation (295–385 nm) were measured with a LI-COR LI-250A light meter (LI-COR, Inc., Lincoln, Nebraska) and an Eppley Lab total UV radiometer (Eppley Laboratory, Inc., Newport, Rhode Island), respectively. Photosynthetically active and UV radiation were 316 ± 39 and 6.8 ± 0.7 μmol s^{-1} m^{-2}, respectively, in fair weather at mid-afternoon. When the saplings reached a height of approximately 100 cm, they were moved to the flat roof top of the four-storey Natural Science building on the University of Michigan campus. Saplings were watered daily and fertilized with Osmocote 15-9-12 slow-release fertilizer (Hummert International, Earth City, Missouri) (50 mL per pot). Mid-afternoon temperatures in fair weather on the roof top site averaged 28.3 ± 1.0 °C. PAR and UV radiation at mid-afternoon were 1545 ± 91 and 87.0 ± 0.6 μmol s^{-1} m^{-2}, respectively. Outdoor UV and wind speed data were collected with a total UV radiometer and an R. M. Young Wind Monitor (R. M. Young Co., Traverse City, Michigan), respectively. They were mounted on the flat roof of the Department of Atmospheric, Oceanic and Space Sciences, approximately 2 km from the site of the poplars. Wind speeds were 1.3 ± 0.04 m s^{-1}, with hourly maxima of 4.5 m s^{-1}. Wind gusts were sufficiently strong to snap off saplings that were not reinforced with stakes. Saplings grew over 120 cm in outdoor conditions, producing 45–47 new leaves, before they were used in a feeding experiment in July 2013.

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). Leaves for feeding experiments were cut through the petiole with a sterile razor blade, using LPI numbers 9–12 for immature leaves, and LPI numbers 17–20 for mature leaves. This cutting method was expected to avoid the induction of defences during the course of the experiment (Osier *et al*., 2000). The immature leaves from LPI 9–12 had recently reached full expansion but retained the same light green colour of the youngest expanding leaves. Chemical and nutritional analyses also confirm that these leaves were immature (see below). Leaves were collected from a subset of four saplings in the mid-morning in July 2013, and their petioles were placed in tubes of water to maintain turgor pressure in the laboratory. Leaf disks were cut with a cork borer (diameter 2.5 cm), avoiding the midrib. Disks were mixed within age groups, and kept in a Petri dish (humidified with a moistened paper towel) for approximately 1 h before they were used for experiments. Representative subsamples of the mixed leaf disks from each day and leaf age were frozen (−80 °C) and lyophilized to measure foliar nutritional quality.

From 5–8 August 2013, the poplar saplings were used to harvest a second set of mature leaves (approximate LPI 17–23). Along with these leaves, the leaves of aspen, cottonwood and willow were sampled from wild trees ($n = 3$–6 per species) at the Kuebler Langford and Bird Hills Nature Areas in Ann Arbor, Michigan. Leaves were selected based on minimal damage, full maturity and full to partial sun exposure. Individual poplar and aspen leaves were cut through the petiole from saplings (height 2–3 m) using a razor blade, and immediately placed in tubes of water. Whole branch tips were cut from cottonwood and willow trees using a pole pruner, and the branches were placed in flasks of water. All leaves were collected in the morning, processed and fed to larvae within a 3-h period. Leaves were washed by soaking (10 min) and gentle rubbing in a soap water solution, rinsed (10 min) and dried with paper towels. Leaf disks were cut as described above, with the exception that 'disks' from the narrow, lanceolate leaves of willow included the midrib.

Effect of leaf age

The amount of leaf tissue consumed and the amount of frass produced by each individual were measured on a dry mass basis (Waldbauer, 1968). The abilities of *L. dispar* larvae to assimilate nutrients from immature or mature poplar leaves were compared. Newly-moulted fourth-instar larvae ($n = 15$ per treatment) were allocated at random between leaf age treatments beginning on 15 July 2013. Larvae were weighed and placed in 30-mL plastic snap-cap cups. Initial larval dry weights were estimated from fresh weight (FW) : dry weight (DW) conversion factors, based on the average FW : DW ratios of five representative larvae. Two leaf disks were weighed and fed to experimental larvae each day during a 3-day feeding period. The use of leaf disks permitted accurate measurements of consumption by providing amounts of food that would be largely consumed (Schmidt & Reese, 1986). A moistened paper filter disk was placed at the bottom of each cup to maintain a humid atmosphere. FW : DW conversion factors were obtained daily for leaf disks from immature and mature leaves ($n = 5$ per age) by weighing them before and after drying (70 °C). Uneaten food and frass were collected from each larva daily. Uneaten food was dried and weighed to determine food consumption, and frass was stored separately for each larva (−80 °C) until it was lyophilized. At the end of the experiment, larvae were kept in empty cups for 6 h to collect their final frass pellets. Larvae were then frozen and dried to determine their final weights. Nutrient assimilation efficiencies were calculated as (ingested nutrient mass − egested nutrient mass)/ingested nutrient mass × 100. Relative protein assimilation rates (RPAR) and relative carbohydrate assimilation rates (RCAR) were calculated as (ingested nutrient mass − egested nutrient mass)/day/initial larval dry mass. Relative protein consumption rates (RPCR) and relative carbohydrate consumption rates (RCCR) were calculated as ingested nutrient mass/day/initial larval dry mass. Relative growth rates (RGR) and relative consumption rates (RCR) were also calculated using initial larval dry masses.

Effect of tree species

The efficiencies with which *L. dispar* larvae assimilate nutrients from the mature leaves of poplar, aspen, cottonwood and

willow were measured. Only measurements from larvae on the mature leaves of each species were necessary to test the main hypothesis regarding PAE, given that a 'high PAE efficiency' of approximately 70% or more had been established previously in *L. dispar* larvae on the leaves of poplar, red oak and sugar maple (Barbehenn *et al*., 2010, 2013, 2014). Newly-moulted fourth-instar larvae ($n = 13$ per tree species) were allocated at random on 5 August 2013 to feed on poplar, aspen, cottonwood or willow. Leaves from each species were collected daily, as described above. Between two and three leaf disks were weighed and fed to each larva on each of 4 days. Food consumption by individual larvae was determined as described above. Final frass pellets were also collected as described above, with the exception that larvae were kept overnight in empty cups to collect their final frass pellets. Larvae were frozen and dried (70 °C, 2 days) to determine their final masses. Nutritional indices were calculated as described above.

Nutritional analysis

Lyophilized leaf disks and frass were ground with a Retsch MM 301 mixer mill (Verder Scientific Inc., Newtown, Pennsylvania) for chemical analysis. Nutrients were analyzed as described previously (Barbehenn *et al*., 2014). Briefly, protein was measured with high performance liquid chromatography as total amino acids (peptide-bound plus free) in acid-hydrolysed samples (4 mg). Sugars (combining glucose, fructose and sucrose) were measured in 10-mg samples with an enzymatic method (Zhao *et al*., 2010). Starch was measured as total glucose in the sugar-extracted samples after enzymatic hydrolysis (Zhao *et al*., 2010). Sugar and starch results were combined as a measure of total nonstructural carbohydrates. Fibre composition was measured in 40-mg samples using neutral detergent fibre, acid detergent fibre and lignin assays, as described previously (Barbehenn *et al*., 2014).

All analytes were measured on a %DW basis, with the exception of water. Water was measured in leaf disks by weighing them before and after they were oven dried (70 °C). Leaf density was calculated as mg (DW) per 4.16 cm^2 (the leaf disk surface area). Because of the irregular shapes of leaf 'disks' from willow, density was not estimated for this species.

Statistical analysis

Foliar nutritional variables were compared by one-way analysis of variance (ANOVA) (SAS Institute, 2010). Replicates within leaf age groups and species were days ($n = 3$ or 4), which limited the statistical power of comparisons of nutritional quality. However, these data were collected primarily to quantify the amounts of nutrients ingested in feeding experiments, rather than to test the main hypotheses. Individual larvae ($n = 10–15$ per treatment) were used as replicates in the feeding experiments. Protein and carbohydrate assimilation efficiencies were analyzed by analysis of covariance (ANCOVA), using egested nutrient mass as the dependent variable and ingested nutrient mass as the covariate (SAS Institute, 2010). Consumption and assimilation rates were analyzed with ANCOVA, using initial larval dry mass as the covariate. The requirement for parallel regression slopes for ANCOVA was confirmed with an analysis of the treatment × covariate interaction term. Where the assumptions of ANCOVA could not be met, ANOVA was used to compare relative consumption and relative growth rates of larvae across treatment groups. Several tests for normality were performed, including the Shapiro–Wilk, Kolmogorov–Smirnov and Anderson–Darling tests (SAS Institute, 2010). Where necessary, data were log transformed to meet the assumptions of ANOVA and ANCOVA.

Results

Immature versus mature poplar leaf quality

Outdoor environmental conditions had a major effect on the leaf morphology of poplar: mature leaves were half the size (52–53%) and 2.6- to 2.8-fold as dense as greenhouse-grown leaves from previous studies (Barbehenn *et al*., 2007, 2010). The leaves on outdoor saplings had red-brown tinged leaf tips, leaf edges and major veins (especially at LPI < 12), rather than having uniformly green leaves with yellow–green veins when grown in a greenhouse. The mature leaves of outdoor saplings contained 13% less carbohydrates but no less protein than immature leaves (Table 1). As expected, mature leaves contained 4% lower water levels and 14% more fibre, and were also 20% denser, than immature leaves. The fibre components that increased significantly during leaf maturation were hemicellulose (increasing from 10% to 11%; d.f. = 1,4; $F = 16.87$; $P = 0.015$) and cellulose (increasing from 9% to 11%; d.f. = 1,4; $F = 21.08$; $P = 0.010$) but not lignin, which was in the range 5–6% (d.f. = 1,4; $F = 2.88$; $P = 0.165$).

Nutrient assimilation from immature versus mature poplar leaves

A major finding was that PAEs in *L. dispar* larvae were equally high on immature and mature leaves (Table 2). Nonetheless, larval growth rates were 25% higher on immature leaves ($P < 0.005$). The growth rates of larvae were strongly and positively associated with their consumption rates ($r^2 = 0.70$), leading to more rapid growth on immature leaves. High consumption rates of immature leaves increased the consumption rates of nutrients, such as protein, producing higher protein assimilation rates (RPAR) in larvae on immature leaves ($r^2 = 0.89$). RPARs were, in turn, strongly associated with larval growth rates ($r^2 = 0.70$). Two other factors that potentially affect RPAR (foliar protein level and PAE) varied little between immature and mature leaves, and therefore played only a minor role in determining RPAR.

As with protein, the assimilation efficiencies of carbohydrates were not significantly affected by leaf maturation (Table 3). As expected, sugars were efficiently assimilated (approximately 95%), whereas starch assimilation efficiencies were

Table 1. Water, protein and fibre levels in immature and mature poplar (*Populus alba* × *Populus tremula* hybrid) (717) leaves from saplings that were grown outdoors.

Leaf maturity	Protein (%DW)	Sugars (%DW)	Starch (%DW)	Total carbohydrate (%DW)	Water (%FW)	Fibre (%DW)	Density (mg cm^{-2})
Immature	15.0 ± 1.1^{a}	10.1 ± 0.2^{b}	4.1 ± 0.3^{a}	14.2 ± 0.2^{b}	67.1 ± 0.2^{b}	24.2 ± 0.4^{a}	5.6 ± 0.2^{a}
Mature	15.5 ± 0.4^{a}	9.0 ± 0.3^{a}	3.4 ± 0.2^{a}	12.4 ± 0.1^{a}	64.7 ± 0.5^{a}	27.7 ± 0.7^{b}	6.7 ± 0.4^{b}
	$P = 0.725$	$P = 0.038$	$P = 0.135$	$P = 0.009$	$P = 0.012$	$P = 0.012$	$P = 0.048$

Data are presented as the mean ± SE, $n = 3$ per maturation stage for all measurements. Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$). DW, dry weight.

Table 2. Consumption and assimilation of protein by fourth-instar *Lymantria dispar* larvae and growth rates on the young or mature leaves of poplar (*Populus alba* × *Populus tremula* hybrid) (717).

Leaf maturity	Relative consumption rate (mg mg^{-1} day^{-1})	Total assimilation efficiency (%)	Relative protein consumption rate (mg day^{-1})	Protein assimilation efficiency (%)	Relative protein assimilation rate (mg mg^{-1} day^{-1})	Relative growth rate (mg mg^{-1} day^{-1})
Immature	2.90 ± 0.09^{b}	31.2 ± 1.4^{a}	0.435 ± 0.013^{b}	75.4 ± 1.0^{a}	0.328 ± 0.011^{b}	0.296 ± 0.013^{b}
Mature	2.39 ± 0.09^{a}	27.7 ± 1.8^{a}	0.371 ± 0.014^{a}	74.8 ± 1.2^{a}	0.277 ± 0.011^{a}	0.236 ± 0.019^{a}
	$P < 0.001$	$P = 0.208$	$P < 0.001$	$P = 0.271$	$P < 0.001$	$P = 0.005$

Data are presented as the mean ± SE, with $n = 11–13$ for all measurements. Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$). Assimilation = (mass ingested − mass egested).

substantially lower (approximately 40%). Higher consumption rates by larvae on immature leaves also led to significantly higher rates of carbohydrate consumption ($r^2 = 0.93$) and assimilation ($r^2 = 0.82$), and RCAR was associated with RGR ($r^2 = 0.60$).

Mature leaf quality in four species of Salicaceae

The nutritional quality of the mature leaves of poplar, aspen, cottonwood and willow were compared (Table 4). Protein was in the range of 15–18% (DW) and was highest in the leaves of willow. This compares with an optimal protein level of 18–20% for *L. dispar* on an artificial diet (Stockoff, 1993). P : C ratios ranged from 1.0 in poplar to 2.0 in willow. Total carbohydrates varied across species from 9% to 15%, with poplar having the highest levels of carbohydrates. However, the proportions of total carbohydrates present as sugars or starch varied, with aspen and cottonwood carbohydrates composed of 85% sugars but poplar and willow carbohydrates composed of 50% and 60% sugars, respectively. In addition, aspen leaves contained significantly less water (by 6%), as well as 20% less fibre, than the other species. Cottonwood leaves were unusually stiff (R. V. Barbehenn, unpublished observations), which was reflected in their high leaf density. However, the lignin content of the four species did not differ significantly, and ranged from 6% to 7% (d.f. = 3, 12; $F = 1.08$; $P = 0.395$). Instead, hemicellulose (ranging from 7% to 12%) and cellulose (ranging from 7% to 10%) were at higher levels in species with higher levels of fibre (d.f. = 3,12; $F = 24.51$; $P < 0.001$ and d.f. = 3,12; $F = 7.69$; $P = 0.004$, respectively). Willow leaf nutritional quality was presumably underestimated by the inclusion of the midrib in leaf samples from this species because larvae did not consume this fibre-rich structure.

Nutrient assimilation from four species of Salicaceae

A major finding in the present study was that *L. dispar* larvae were able to assimilate protein with high efficiencies (68–76%) from the mature leaves of all four species of Salicaceae (Table 5). Between the two macronutrients measured, RPAR was more strongly associated with growth rate ($r^2 = 0.92$) than was RCAR ($r^2 = 0.41$). RPAR varied by almost three-fold between larvae on the four species. This variation was explained largely by protein consumption rate ($r^2 = 0.99$). In turn, RPAR was strongly associated with the overall consumption rate ($r^2 = 0.98$). By contrast, the protein content of the leaves had less impact on RPCR ($r^2 = 0.34$, driven entirely by the willow treatment group). Therefore, larvae that fed at higher rates had higher growth rates ($r^2 = 0.95$).

Carbohydrate assimilation efficiencies (CAE) in *L. dispar* on cottonwood, poplar and willow were in the range of 72–77% but, on aspen, CAE reached 92% (Table 6). The high CAE in larvae on aspen appeared to be a result of the predominance of sugars in its leaves, and the extremely efficient assimilation of sugars. Therefore, it was unexpected that sugar assimilation efficiencies were more than 10% lower on cottonwood than on the other species ($P < 0.001$). As found previously, *L. dispar* larvae were relatively inefficient at assimilating starch (Table 6). Leaves of species of Salicaceae that contain higher proportions of sugars have assimilated total carbohydrates more efficiently (Fig. 1).

The P : C ratios in the leaves of the four species of Salicaceae were strongly associated with the P : C assimilation rates in larvae that fed on each of these species ($r^2 = 0.80$). Data for larvae of *L. dispar* feeding on the four species of Salicaceae are presented with data from larvae on greenhouse-grown poplar (R. V. Barbehenn, N. Haugberg, J. Kochmanski & B. Menachem, unpublished observations) to show that the same

Table 3. Consumption and assimilation of carbohydrates by fourth-instar *Lymantria dispar* larvae on the immature or mature leaves of poplar (*Populus alba* × *Populus tremula* hybrid) (717).

Leaf maturity	Relative carbohydrate consumption rate (mg mg^{-1} day^{-1})	Sugar assimilation efficiency (%)	Starch assimilation efficiency (%)	Carbohydrate assimilation efficiency (%)	Relative carbohydrate assimilation rate (mg mg^{-1} day^{-1})
Immature	0.412 ± 0.012^{b}	95.3 ± 0.3^{a}	40.3 ± 4.9^{b}	78.6 ± 1.7^{a}	0.324 ± 0.011^{b}
Mature	0.297 ± 0.011^{a}	94.5 ± 0.4^{a}	36.8 ± 4.5^{a}	77.9 ± 1.6^{a}	0.227 ± 0.011^{a}
	$P < 0.001$	$P = 0.198$	$P = 0.039$	$P = 0.804$	$P < 0.001$

Carbohydrates include sugars and starch. Data are presented as the mean ± SE, $n = 11–13$ for all measurements. Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$). Assimilation efficiency = (mass ingested − mass egested)/mass ingested × 100.

Table 4. Protein, carbohydrate, water, and fibre levels and leaf density in the mature leaves of four species of Salicaceae.

Species	Protein (%DW)	Sugars (%DW)	Starch (%DW)	Total carbohydrate (%DW)	Water (%FW)	Fibre[a] (%DW)	Density[b] (mg cm^{-2})
Poplar[c]	15.1 ± 0.5^{a}	7.60 ± 0.44^{b}	7.29 ± 0.92^{c}	14.89 ± 1.29^{b}	63.8 ± 0.7^{b}	25.4 ± 0.5^{b}	7.7 ± 0.1^{a}
Aspen[d]	17.1 ± 0.3^{bc}	8.42 ± 0.57^{b}	1.02 ± 0.23^{a}	9.44 ± 0.76^{a}	60.0 ± 0.7^{a}	20.0 ± 0.6^{a}	7.4 ± 0.2^{a}
Cottonwood[e]	15.9 ± 0.5^{ab}	10.11 ± 0.76^{c}	1.65 ± 0.19^{b}	11.76 ± 0.95^{a}	64.1 ± 0.1^{b}	26.1 ± 0.7^{b}	9.0 ± 0.4^{b}
Willow[f]	18.4 ± 0.9^{c}	5.76 ± 0.22^{a}	3.46 ± 0.44^{c}	9.22 ± 0.52^{a}	63.7 ± 0.9^{b}	23.9 ± 0.4^{b}	—
	$P = 0.010$	$P < 0.001$	$P < 0.001$	$P = 0.003$	$P = 0.002$	$P < 0.001$	$P = 0.008$

[a]Fibre was measured as neutral detergent fibre, which includes cellulose, hemicellulose and lignin but not pectin.
[b]Willow leaf density was not calculated because these narrow leaves were not cut into disks with a fixed area.
[c]Poplar, *Populus alba* × *Populus tremula* hybrid (717).
[d]Trembling aspen, *Populus tremuloides*.
[e]Eastern cottonwood, *Populus deltoides*.
[f]Black willow, *Salix nigra*.
Data are presented as the mean ± SE, $n = 4$ replicate days for all measurements. Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$). DW, dry weight.

relationship exists over a wide range of leaf qualities ($r^2 = 0.97$ for treatment groups presented in Fig. 2). With a slope of 0.87, this relationship indicated that larvae assimilated carbohydrates at a slightly higher rate than protein. By contrast, neither foliar P : C ratios ($r^2 = 0.021$), nor larval RPAR : RCAR ratios ($r^2 = 0.011$; Fig. 2) were associated with larval growth rates. Foliar P : C ratios were only weakly (negatively) associated with consumption rates ($r^2 = 0.27$). As reported above, RCR does explain much of the variation in RGR for *L. dispar* across all of the treatment groups ($r^2 = 0.51$).

Discussion

The present study identifies a potentially important nutritional difference between trees in different families; protein in the mature leaves of species in the Salicaceae remains readily available to *L. dispar* compared with protein in the mature leaves of previously examined tree species in the Fagaceae and Aceraceae. Specifically, the PAE of *L. dispar* larvae remains near 70% from the mature leaves of poplar, aspen, cottonwood and willow, whereas it falls to 45–52% in *L. dispar* on mature red oak and sugar maple (Barbehenn *et al*., 2013, 2014). The changes in PAE are interpreted as largely being a result of protein extractability. Protein in the mature leaves of red oak becomes significantly less extractable in the midguts of *L. dispar* larvae when PAE decreases (Barbehenn *et al*., 2013, 2014), whereas protein in the mature leaves of poplar remains highly extractable and PAE also remains high (R. V. Barbehenn, N. Haugberg, J. Kochmanski & B. Menachem, unpublished observations).

The mechanisms that explain the high availability of protein from the mature leaves of species of Salicaceae, as well as the large decrease in PAE in larvae on the mature leaves of red oak and sugar maple (Barbehenn *et al*., 2013), are unknown. The obvious changes in leaves at maturity involve increases in fibre and other changes in cell wall structure. However, it is already clear that changes in total fibre levels cannot explain the patterns of PAE observed between tree species. For example, red oak leaves produce significant increases in fibre (including lignin and cellulose) during leaf maturation, although sugar maple leaves produce no significant changes in total fibre during leaf maturation (Barbehenn *et al*., 2013, 2014). In addition, increased levels of fibre in the mature leaves of poplar are not associated with decreased protein availability (present study; R. V. Barbehenn, N. Haugberg, J. Kochmanski & B. Menachem, unpublished observations). Other differences between species with high or low protein availability may include the extent of cross-linking of cell wall components, the pectin content of cell walls and/or plasmodesmata frequency (i.e. wall porosity). In the present study, the first two possibilities are not measurable via the methods used for determining fibre composition. Indeterminate (continuously growing) species do have a high frequency of plasmodesmata in their cell walls where a rapid flux of photosynthate between the mature (source) and immature (sink) leaves occurs (Turgeon, 1989; Patrick, 1997; Roberts *et al*., 2001). Clearly, there is a need to understand

Table 5. Consumption and assimilation of total dry mass and protein by fourth-instar *Lymantria dispar* larvae and their growth rates on the mature leaves of four species of Salicaceae.

Species	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})
Poplar	3.46 ± 0.09^{c}	28.7 ± 1.3^{a}	0.522 ± 0.014^{c}	73.9 ± 0.9^{bc}	0.385 ± 0.010^{c}	0.419 ± 0.017^{c}
Aspen	1.75 ± 0.12^{a}	26.7 ± 1.8^{a}	0.300 ± 0.021^{a}	69.1 ± 1.3^{ab}	0.207 ± 0.014^{a}	0.160 ± 0.015^{a}
Cottonwood	2.61 ± 0.10^{b}	25.1 ± 1.5^{a}	0.414 ± 0.016^{b}	68.4 ± 1.0^{a}	0.283 ± 0.012^{b}	0.308 ± 0.016^{b}
Willow	4.84 ± 0.16^{d}	27.7 ± 0.8^{a}	0.890 ± 0.030^{d}	76.0 ± 0.7^{c}	0.676 ± 0.024^{d}	0.630 ± 0.024^{d}
	$P < 0.001$	$P = 0.160$	$P < 0.001$	$P = 0.006$	$P < 0.001$	$P < 0.001$

Data are presented as the mean ± SE, $n = 10–13$ for all measurements. Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$).

Table 6. Consumption and assimilation of carbohydrates from the mature leaves of four species of Salicaceae by fourth-instar *Lymantria dispar* larvae.

Species	Relative carbohydrate consumption rate (mg mg^{-1} day^{-1})	Sugar assimilation efficiency (%)	Starch assimilation efficiency (%)	Total carbohydrate assimilation efficiency (%)	Relative carbohydrate assimilation rate (mg mg^{-1} day^{-1})
Poplar	0.489 ± 0.015^{d}	95.8 ± 0.4^{b}	46.7 ± 4.7^{a}	71.6 ± 2.2^{a}	0.365 ± 0.013^{c}
Aspen	0.160 ± 0.012^{a}	96.8 ± 0.6^{c}	57.1 ± 4.9^{c}	92.5 ± 0.9^{c}	0.153 ± 0.011^{a}
Cottonwood	0.305 ± 0.013^{b}	83.7 ± 1.6^{a}	41.4 ± 4.3^{b}	77.2 ± 1.8^{b}	0.244 ± 0.012^{b}
Willow	0.434 ± 0.013^{c}	94.0 ± 0.9^{b}	50.1 ± 4.5^{a}	77.4 ± 1.7^{ab}	0.347 ± 0.016^{c}
	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$	$P < 0.001$

Data are presented as the mean ± SE, $n = 10–13$ for all measurements. Summary statistics followed by different superscript lowercase letters are significantly different ($P < 0.05$). Assimilation efficiency = (mass ingested − mass egested)/mass ingested × 100.

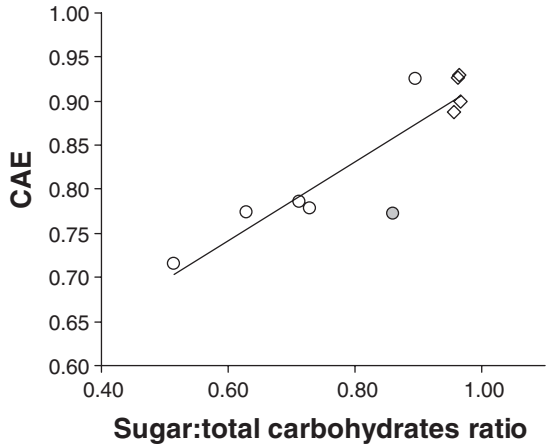

Fig. 1. Association between the proportion of sugar in total foliar carbohydrates and carbohydrate assimilation efficiencies (CAE) of *Lymantria dispar* larvae. Data plotted include the means from larvae on immature and mature poplar and mature aspen, cottonwood, and willow (circles) and greenhouse-grown poplar (diamonds; R. V. Barbehenn, N. Haugberg, J. Kochmanski & B. Menachem, unpublished observations) ($r^2 = 0.79$). $r^2 = 0.58$ using the six data points from the present study. The grey circular point represents cottonwood, from which sugars were assimilated at lower efficiencies than from the other species of Salicaceae.

the role of leaf tissue structure in limiting the availability of macronutrients such as protein and starch.

The overall availability of carbohydrates from the mature leaves of all examined species remains relatively high for *L. dispar*: CAEs are in the range of 72–92% from the immature or mature leaves of species in the Salicaceae, and in the range of 68–85% from the immature or mature leaves of red oak and sugar maple (Barbehenn *et al*., 2014; R. V. Barbehenn, unpublished observations). Although sugars are assimilated efficiently from the mature foliage of all tested species (e.g. 82–97%), starch has lower assimilation efficiencies from both immature and mature leaves (e.g. 37–57%) (Barbehenn *et al*., 2014; R. V. Barbehenn, unpublished observations). This pattern of carbohydrate assimilation efficiencies is reported in some previously examined species of caterpillars on tree leaves (Horie *et al*., 1985). Low starch assimilation efficiency may result from a number of factors, including the inability of starch grains to diffuse out of leaf tissues, the recalcitrance of starch grains to enzymatic hydrolysis, and/or low amylase activity in some insect species (Waldbauer, 1968; Harvey, 1975). The higher assimilation efficiency of sugars than starch means that total CAE depends, in part, on the relative proportions of sugars and starch in leaves. For a given level of total carbohydrates, tree species with higher proportions of sugars than starch, such as aspen, are expected to provide more energy for caterpillars than species with greater proportions of starch, such as willow. Although the CAEs of *L. dispar* larvae on aspen, poplar and willow leaves fit this pattern, the relatively low CAE of larvae on cottonwood is unexpected. More commonly, sugars are efficiently assimilated and unneeded amounts are eliminated by increased respiration rates (Zanotto *et al*., 1993; Trier & Mattson, 2003).

Regardless of variation in foliar nutrient composition, most protein and carbohydrate is assimilated with a high efficiency by *L. dispar* larvae on species of Salicaceae. The strong

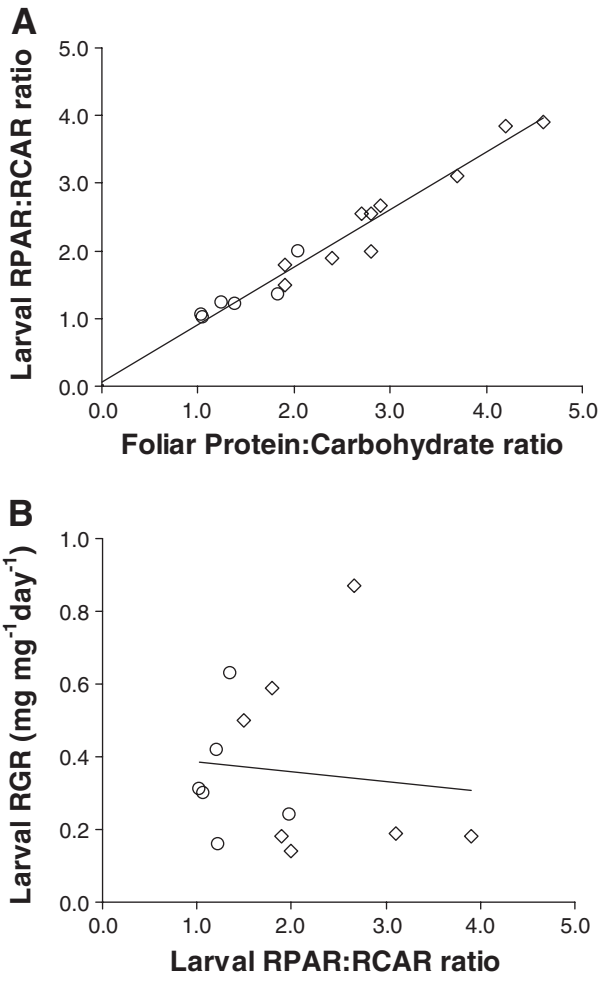

Fig. 2. (A) Association between protein : carbohydrate ratios in the leaves of four species of Salicaceae and the ratios of protein : carbohydrate relative assimilation rates (RPAR : RCAR) in *Lymantria dispar* larvae ($r^2 = 0.97$; slope = 0.87). $r^2 = 0.80$ using the six data points from the present study. (B) Association between RPAR : RCAR ratios and relative growth rate (RGR) for *L. dispar* larvae ($r^2 = 0.011$). Data plotted include the means from larvae on immature and mature poplar and mature aspen, cottonwood, and willow (circles) and greenhouse-grown poplar (diamonds; R. V. Barbehenn, N. Haugberg, J. Kochmanski & B. Menachem, unpublished observations).

association between foliar P : C ratios and larval RPAR : RCAR ratios (Fig. 2) suggests that *L. dispar* larvae maximize PAE and CAE. The digestive strategy of *L. dispar* larvae is generally consistent with 'the prevailing view … that animals maximize the extraction of all macronutrients from food in the [gastrointestinal tract] and then use post-absorptive mechanisms to regulate retention and use of different nutrients' (Clissold *et al*., 2010). It is noteworthy that the relationship between foliar and assimilated nutrient ratios by *L. dispar* larvae on immature red oak and sugar maple leaves ($r^2 = 0.94$; slope = 0.78) is similar to that observed for larvae on species of Salicaceae (Fig. 2). However, on mature oak and maple leaves, foliar and assimilated nutrient ratios are unrelated ($r^2 = 0.14$; slope = 0.20) (Barbehenn *et al*., 2014; R. V. Barbehenn, unpublished observations). These different relationships highlight the nutritional differences between the mature leaves of oak and maple versus species in the Salicaceae. The results of this work not only emphasize the importance of measuring P : C ratios in plants, but also the ratios of their assimilation rates when attempting to understand the availabilities of nutrients from plants. The larvae of *L. dispar* are able to cope with wide variation in foliar P : C ratios, as indicated by the low impact of such variation on their growth and consumption rates.

Leaf consumption rate is strongly associated with larval growth rate, and consumption rate appears to be the single most important factor affecting larval performance. Lower consumption rates by larvae on mature leaves are commonly observed, and are presumed to result from the difficulty of ingesting more fibrous and tough mature leaves (Hough & Pimentel, 1978; Schweitzer, 1979; Schroeder, 1986; Hunter & Lechowicz, 1992; Choong, 1996; Parry *et al*., 1998; Kursar & Coley, 2003). Thus, compensatory feeding by insect herbivores on mature leaves may be impossible (Clissold *et al*., 2009; Barbehenn *et al*., 2013). By contrast, high leaf consumption rates, coupled with high nutrient assimilation efficiencies, result in high nutrient assimilation rates. As noted above, RPAR is strongly associated with growth rates, whereas RCAR appears to have a smaller effect on growth rates than does RPAR. In addition, foliar protein levels have only a minor effect on RPAR, as found in *L. dispar* larvae on immature and mature red oak leaves (Barbehenn *et al*., 2014).

The potential effects of environmental stresses during leaf development on nutrient availability to herbivores are also addressed in the present study. The differences in leaf morphology and nutritional quality between poplar grown in a greenhouse or outdoors are dramatic, as expected between sun and shade leaves (Coley & Barone, 1996). Nevertheless, there are no significant decreases in protein or carbohydrate availabilities associated with poplar leaf maturation in either greenhouse or outdoor conditions (present study; R. V. Barbehenn, N. Haugberg, J. Kochmanski, B. Menachem, unpublished observations).

The results of the present study are relevant to a central area in plant–herbivore theory: the relationship between insect performance and host plant selection. Greater performance (e.g. growth rate or body mass) on certain plant species would increase insect fitness (Hough & Pimentel, 1978; Awmack & Leather, 2002), which should drive the evolution of host preference (Jaenicke, 1990). If the mature leaves of trees in the Salicaceae are more nutritious than those from many other species, then insect herbivores might be expected to favour the Salicaceae as host plants. Although species of Salicaceae are among the top host plants for *L. dispar*, many oak species are also top host plants, indicating that determinate growth and a rapid drop in protein availability in mature leaves are not sufficient to constrain the host range of this generalist herbivore. Tree-feeding Macrolepidoptera, such as *L. dispar*, cannot complete their larval development before the leaves on their host plants reach maturity. Indeed, most larval consumption occurs on mature leaves by *L. dispar* during its final instar, despite having begun larval development during the first week after budburst (Raupp *et al*., 1988; Hamilton & Lechowicz,

1991). Thus, the results of the present study suggest that generalist Macrolepidoptera have a choice between tree species that all have lower nutritional quality after their leaves mature but differ in the extractability of foliar protein.

In conclusion, the results of the present study emphasize the importance of protein availability from mature tree leaves for the fitness of Macrolepidoptera such as *L. dispar*. Because *L. dispar* larvae must complete their development on mature foliage, the present study suggests one reason why species of Salicaceae are among the top host plants for this herbivore: they provide readily available protein throughout the growing season. The results of the present study also highlight the different patterns of change in protein availability among trees in different families during leaf maturation; PAE either declines rapidly at leaf maturity (e.g. some species in the Fagaceae and Aceraceae) or remains high (e.g. species in the Salicaceae). In addition, CAEs are greatest in larvae that feed on low starch-, high sugar-containing leaves because sugars are more efficiently assimilated than starch. Further work is needed to determine whether tree species with indeterminate growth generally have highly available foliar protein, or whether this is an unusual trait shared by species in the Salicaceae.

Acknowledgements

We thank Gary Phillips and Damon Place for technical assistance on the roof; Christine Lokerson and Hannah Nadel (USDA) for *L. dispar* eggs; Zachary Ebenstein for assistance with meteorological measurements; and the University of Michigan Program in Biology for Underwood-Alger Scholarships (for JK and WN) and a K.L. Jones Award (for WN).

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Accepted 7 December 2014
First published online 2 January 2015