Nutrient cycling by the herbivorous insect *Chrysomela tremulae*

Nutrient content in leaves and frass and measurements of ingestion, egestion and exuviation rates

Sara Anderson

Nutrient cycling by the herbivorous insect *Chrysomela tremulae*

Sara Anderson

Abstract

Insect herbivory on forest canopies strongly affects ecosystem nutrient cycling and availability in a variety of ways, e.g. by changing the quantity, quality and timing of nutrient input to forest soils. A qualitative method for measurements of ingestion, egestion and exuviation rates of the insect *Chrysomela tremulae* on leaves of the hybrid *Populus tremula* x *tremuloides* were tested in this study with the aim to detect differences in relative nutrient cycling efficiencies. The assimilation efficiency (AD), efficiency of conversion of digested food (ECD) and efficiency of conversion of ingested food (ECI) increased from 1st, through 2nd and 3rd instar larvae with generally higher efficiencies for nitrogen than carbon. Effects on nutrient limitations for the insect were also tested by increasing the C:N ratio of insect diet. A carbohydrate solution was painted onto leaves which resulted in a significant increase in C:N ($p<0.0001$). This lead to a trend of lengthened developmental time for each ontogenetic stage, as well as higher ingestion rate and lower egestion- and exuviation rates. However, a different method of increasing the C:N ratio is recommended in future experiments since the leaves never truly absorbed the solution.

Key Words: *Insect herbivory, nutrient cycling efficiencies, approximate digestibility, Chrysomela tremulae, increased C:N.*

List of content

1. Introduction

1.1 Nutrient cycling and herbivory

The idea that herbivores play a role in the regulation of primary productivity and nutrient cycling originated during the 1960s (Hunter 2001). The mechanisms by which herbivores influence nutrient cycling in an ecosystem can be categorized as part of either the slow or the fast cycle, as stated by McNaughton et al. (1988). The slow cycle includes release of nutrients from plant litter while the fast cycle includes release of nutrients from faecal droppings and dead herbivores. The fast cycle recycles and releases nutrients for plant uptake more rapidly than the slow cycle. Herbivores play a role in the slow cycle by altering the quantity and quality of plant litter when they prefer to feed on plants that differ in litter decomposition rates (Pastor & Naiman 1992). Preferential feeding by herbivores on plants that produce litter with a slower decomposition rate can speed up the slow cycle. On the other hand, if herbivores feed on plants with fast decomposing litter, it further reduces the slow cycle (Belovsky & Slade 2000). If the proportion of nutrients released by the fast or the slow cycle shifts, it can change the nutrient availability for plants. This can in turn modify plant species composition and productivity in a community. If the deleterious effects of plant consumption are outweighed by preferential feeding on slowly decomposing plants, the nutrient cycling can accelerate and increase plant production (Pastor & Naiman 1992). If plant species composition is altered due to changes in nutrient cycling, further changes in nutrient cycling, plant production and species composition is to be expected, thus creating a positive or self-enhancing feedback (Belovsky & Slade 2000). Today, the idea that moderate rates of herbivory increases nutrient cycling rate is a widespread and accepted theory (Seastedt & Crossley 1984; Hunter 2001).

1.2 Insect herbivory

Insect herbivores strongly influence terrestrial cycling of nutrients, mainly due to their rapid turnover and high abundance (Seastedt & Crossley 1984). Mattson and Addy (1975) even argued that insect herbivores have a larger impact on ecosystems than detritivores, because detritivores feed on non-living detritus while herbivores feed on living plants. The detritivore-detritus interaction has less potential of affecting the ecosystem in both negative and positive feedback loops than herbivore-plant interactions. Another aspect when it comes to insect herbivores and their role in nutrient cycling includes their propensity for periodic outbreaks. These outbreaks often result in very different outcomes of nutrient inputs than what is seen otherwise (Hunter 2001). Lovett and Ruesink (1995) showed that during outbreaks with complete defoliation the nutrient cycles, soil water relations and microbial activity were severely altered. Furthermore, insects are perfect study animals when it comes to their participation in nutrient cycling because of their short life span, small size and limited home range.

Insects need to ingest a balanced diet of nutrients in order to survive and reproduce; these include amino acids, sterols, phospholipids, carbohydrates, fatty acids, minerals, vitamins, trace elements and water (Behmer 2009). Plants contain all these essential nutrients, but the ratios and absolute amounts of these can be very variable. The variation is highest between different plant species, but also within species due to differences in genetic and environmental conditions. Different parts of plant individuals also vary in nutrient content, e.g. between young and mature leaves (Behmer 2009). The nutritional constituents of the ingested diet of the insects may not be 100% digested and absorbed. The amount that is digested is denoted by the assimilation efficiency (AD). Parts of the nutrients digested are lost as work or respiration, but the proportion that is assimilated into net biomass is denoted by the efficiency of conversion of digested food (ECD). A third parameter denotes the efficiency of conversion of ingested food (ECI). To summarize, AD is the digestibility of food, whereas ECI and ECD is how efficient an insect converts that food into biomass (Scriber & Slansky 1981).

Insect herbivory on forest canopies strongly affects the ecosystem nutrient cycling and availability in a variety of ways, e.g. by changing the quantity, quality and timing of nutrient input to forest soils (Belovsky & Slade 2000; Madritch et al. 2007). Seastedt and Crossley (1984) stated that herbivory by arthropods in canopies have the greatest consequence on mobile elements like potassium, while detrivores in the soil community have greater effect on less mobile elements like nitrogen, phosphorus and calcium. However, insect herbivory still have effects on less mobile nutrients. Frost and Hunter (2004) demonstrated that frass deposition by insect herbivores increased soil nitrogen (N) and carbon (C) pools, but also increased soil respiration and leaching of nitric oxide, thus having both direct and indirect effects on nutrient dynamics. Frass deposition from insects can be of notable quantities, and studies shows that the return of N to soils from faeces can exceed that from plant litter (Fogal & Slansky 1985) and the rate of N return to soil from plants can be doubled (Hollinger 1986). Insects can thus increase the proportion of nutrients released by the fast cycle compared to the slow cycle, and hence change the nutrient availability in a community.

Insect defoliation can increase phenolic compounds in the leaves (Findlay et al. 1996) and dead insect bodies that are deposited on the ground contain more easily decomposed nutrients than leaf litter (Schowalter et al. 1986). This nutrient input can in turn stimulate litter decomposition during insect outbreaks (Seastedt & Crossley 1984). The nutrient content of precipitation that passes through the canopy is higher when canopies are subject to insect defoliation. Leaching of nutrients is bigger from damaged leaves (Tukey & Morgan 1963), but the precipitation also dissolves faeces in the foliage. Increased greenfall due to insect herbivory affects the decomposing fauna and thus the nutrient cycling (Bradford et al. 2008).

Finally, insect defoliation impacts the structure of plant canopies, thus altering the cover and light availability to underlying communities. This in turn influences the soil microclimate (temperature and moisture) and ultimately nutrient cycling (Mulder 1999).

1.3 Carbon and nitrogen in leaves

An increase in the carbohydrate content of insect diet would alter the C:N ratio, with possible effects on nutrient limitations for the insect. This could be investigated in insect feeding trials with experimental addition of carbohydrates on leaves. One expectation from increased C content is a prolonged development of each ontogenetic stage because the increase in C will create a scarcity of N. N deficiency decreases the growth of most herbivorous insects which consequently leads to higher feeding rates and subsequently decreases the assimilation efficiency (Scriber 1977; Berner, Blanckenhorn & Körner 2005). Another implication is that lengthened development leads to an increased exposure to predation and parasitism. Longer development also reduces the turnover and inputs like exuvia and dead bodies to the fast cycle. Also, an increase in C to N leads to a need for the insects to excrete the excess C and ultimately to an increase in frass deposition. However, the frass will contain less N and this could alter the nutrient composition in the soil and possible nutrient limitation for other organisms.

Insect herbivores play a significant role in nutrient cycling, they are both affected by the nutrient content in the diet, but also affect the return of nutrients to the soil. This study focus on insect growth efficiency as a function of plant nutrient content.

1.4 Aim

This study aimed to qualitatively test methods for measurements of ingestion, egestion and exuviation rates in a feeding trial with the insect *Chrysomela tremulae* on leaves of the hybrid *Populus tremula* x *tremuloides*. A method to increase C:N in leaves was tested and C and N content of leaves and faeces was analyzed with the aspiration to detect differences in relative nutrient cycling efficiency. If insect herbivores recycle nutrients with different

efficiencies, there is a possibility that they can affect the plant community composition, since plant growth and competition depend on soil nutrient availability.

2. Method

2.1 Study organism

The poplar leaf beetle, *Chrysomela tremulae* (Coleoptera: Chrysomelidae), is a common defoliating pest of *Populus tremula,* both when it comes to young plants and short rotations stands in Europe (Augustin et al. 1993). It is a polyvoltine insect, meaning it has more than four generations during a year or a season. There are eight developmental stages of *C. tremulae*; egg (Figure 1), 1st instar larvae (L1), 2nd instar larvae (L2), 3rd instar larvae (L3), prepupa, pupa, imago and adult, and during natural conditions, the length of each stage is a direct function of the mean ambient temperature (Augustin & Lévieux 1993). Batches of eggs are laid on the lower side of leaves (Gruppe et al. 1999) and the L1 feeds and makes windows in the leaves close to where they hatched (Figure 2) (Augustin et al. 1993). The larvae then molt to 2nd and 3rd instar larvae with increasing activity and dispersal over the foliage (Figure 3 and 4). The beetle pupates on the foliage (Augustin & Lévieux 1993). First it exists in a prepupae form where it prepares for the pupae stage. During this phase no eating occurs as it searches for a suitable place (Figure 5). The exuvia of the final instar larvae remains attached to the abdominal tip of the pupa and anchor it to the foliage during pupation (Figure 6) (Jolivet et al. 1988). The imago stage includes maturing of the beetle, during which no feeding occurs and the colors deepens as it reaches the adult reproducing stage (Figure 7 and 8). The adults disperse over the aspens and perform hole feeding. During heavy outbreaks larvae and beetles only leave the veins of the leaves, and adults can also feed on the bark when foliage becomes scarce, which eventually kills the seedlings (Leplé et al. 1995).

Figure 1. Newly laid eggs.

Figure 2. Feeding 1st instar larvae.

Figure 3. Molt between 2nd and 3rd instar larvae, 10x magnified.

Figure 4. Feeding 3rd instar larvae.

Figure 5. Prepupa attached to an Eppendorf tube.

Figure 6. Pupating beetle, here attached to the cage net.

Figure 7. Imago just emerged from pupae.

Figure 8. Mating adult beetles.

The *C. tremulae* used in this study were from a laboratory colony that had been kept for three years and outcrossed with newly collected individuals each spring. The insects were reared on aspen foliage in cages at 21.5°C, with relative humidity (RH) of 75% and a 15 h photoperiod.

2.2 Leaf preservation method

Leaves were collected from 12 individuals of *P. tremula* x *tremuloides* (T89) that had grown in greenhouse conditions. In order to prevent leaves from desiccation, a method to maintain fresh leaves for several days in petri dishes was tested. Leaf area and fresh weight of each leaf was premeasured. The leaf area was measured in an area meter (LI-3000C Portable Area Meter, LI-COR Inc.). The petiole of each leaf was cut and put in Eppendorf tubes with 2 mL distilled water to maintain turgor and prevent water loss (Figure 9). Water was refilled in the tubes when needed. After 96 hrs the area and weight was measured again. Five control leaves without tubes were tested for statistical differences with five leaves in tubes.

Figure 9. Leaf in an Eppendorf tube with water.

2.3 Leaf treatment method

In order to perform feeding trials with leaves of different C:N ratios, a carbohydrate solution was painted on the subside of some leaves. Carbohydrates exist in a variety of forms in aspen leaves, but the total soluble carbohydrate (TSC) amount exceeds the starch amount, with sucrose as the dominant form (Mandre et al. 2011; Chow & Landhäusser 2004). Average C content of *P. tremula* x *tremuloides* leaves was estimated from data measurements of 248 leaves (Mehdi Cherif, unpublished data). Additionally 50% C was added per leaf in the form of a sucrose solution. Each leaf was painted with 10 µL/cm^2 solution and the solution contained 160.788 g/L sucrose ($C_{12}H_{22}O_{11}$) and 50% acetone (see section 2.3.1 Sucrose concentration calculation). Dry weight (DW) percentages of leaves were estimated from FW and DW measurements of 10 leaves that were weighed before and after 24 hrs freeze drying (CoolSafe 95/55-80, ScanVac, LaboGene™).

The solution was pipetted onto the leaf and spread with a fine brush that had been dipped in 100% acetone to prevent any solution to be absorbed by the hair on the brush (Figure 10). Two types of control leaves were used, unpainted leaves and leaves painted with 50% acetone only and with a different brush.

This method has successfully been used by Hemming and Lindroth (2000) and Noseworthy and Despland (2006). In a similar feeding trial with *Epirrita autumnata* (Lepidoptera: Geometridae) on *Betula pubescens*, Salminen and Lempa (2002) showed that the larvae did not eat less from painted leaves compared to control leaves, suggesting that no bias occurred even under no-choice circumstances.

Figure 10. Sucrose solution was pipetted onto leaves and spread with a brush.

2.3.1 Sucrose concentration calculation

Average fresh-weight-based specific leaf area was 73.529 cm²/g (from measurements of 43 leaves of *P. tremula* x *tremuloides*). Average DW percentage of FW in leaves was 21.49% and calculated from 10 leaves with average fresh weight of 0.770 g and average dry weight after 24 hrs freeze drying of 0.167 g. DW/FW was divided by fresh-weight-based specific leaf area to obtain DW per area (Eq. 1).

$$\frac{0.2149}{73.529\ cm^2/g} = 0.002923\ g/cm^2 \qquad\qquad (Eq.\ 1)$$

Average C amount in leaves was 46.33% (estimated from data of 248 leaves in g(C)/g DW leaf, Mehdi Cherif, unpublished data). Each leaf then contains approximately 0.001354 g(C)/cm² (Eq. 2).

$$0.4633 \times 0.002923\ g/cm^2 = 0.001354\ g(C)/cm^2 \qquad\qquad (Eq.\ 2)$$

In order to increase C amount with 50%, 0.00067703 g(C) needs to be added per cm² (Eq. 3).

$$0.5 \times 0.001354\ g(C)/cm^2 = 0.00067703\ g(C)/cm^2 \qquad\qquad (Eq.\ 3)$$

The concentration of the solution was 67.703 g/L if 10 μL solution was to be added per cm² (Eq. 4).

$$\frac{0.00067703\ g(C)/cm^2}{0.00001\ L/cm^2} = 67.703\ g(C)/L \qquad\qquad (Eq.\ 4)$$

The molecular weight of C is 12.011 g/mol. The amount of substance that needs to be added is 5.6367 mol/L (Eq. 5), from n = m/M.

$$n = \frac{67.703\ g/L}{12.011\ g/mol} = 5.6367\ mol(C)/L \qquad\qquad (Eq.\ 5)$$

C has 1 atom C while sucrose has 12 atoms C, hence the amount of sucrose that needs to be added is only a 12th of the amount of sugar in moles (Eq. 6).

$$n = \frac{5.6367\ mol/L}{12\ mol(C)/mol(sucrose)} = 0.4697\ mol(sucrose)/L \qquad\qquad (Eq.\ 6)$$

The molecular weight of sucrose is 342.30 g/mol. Hence, the amount of sucrose that needs to be added is 160.788 g/L (Eq. 7), from m = n*M.

$$m = 0.4697\ mol(sucrose)/L \times 342.30\ g/mol = 160.788\ g/L \qquad\qquad (Eq.\ 7)$$

2.4 Feeding trial

Eggs of *C. tremulae* were put in separate petri dishes (120 by 120 mm), one egg per petri dish, together with one leaf in an Eppendorf tube. The petri dishes were stored at 21.5°C, RH 75% and a 15 h photoperiod. Whenever a beetle transformed into the next developmental stage, it was taken out and FW of the beetle was measured. If the condition of the eaten leaf made it possible, leaf FW and area were also measured. The beetle was then put in a new petri dish together with a new leaf for the next stage and the procedure was repeated during next transformation. Leaves, molts and faeces were collected at every transformation, freeze dried for 24 hrs, molded and weighed and finally sent for C:N analysis (ECS 4010 CHNSO Analyzer, Costech Instruments, Analytical Technologies, Inc.)

2.5 Data calculations

From the data obtained, nutrient uptake through ingestion, nutrient loss through faeces, and assimilation of nutrients in beetles together with ingestion, egestion and exuviation rates were calculated. Carbon and nitrogen content in leaves and faeces were used to detect any effect on relative nutrient cycling.

2.5.1 Ingestion, egestion and exuviation rates

Ingestion rate (unit g(DW)/day) equals the difference in DW of leaves between end and start of a stage, estimated from weighing and measuring the eaten area, divided by number of days of the developmental stage of interest (Eq. 8). Initial DW of leaves (g) was estimated by multiplying the initial area (cm²) with the end DW/area (g/cm²) (Eq. 9). The DW decrease was then obtained by subtracting the end DW from the initial DW (Eq. 10).

$$Ingestion\ rate = \frac{leaf\ DW\ decrease}{no\ of\ days} \qquad \text{(Eq. 8)}$$

$$Initial\ DW = \frac{end\ DW}{end\ area} \times initial\ area \qquad \text{(Eq. 9)}$$

$$DW\ decrease = initial\ DW - end\ DW \qquad \text{(Eq. 10)}$$

Ingestion rate of C or N (g(C or N)/day) is obtained by multiplying the ingestion rate (g/day) with the percent of C or N content in the leaf (g(C or N)/g(DW) (Eq. 11).

$$Ingestion\ rate\ of\ C\ or\ N = ingestion\ rate \times C\%\ or\ N\ \%\ in\ leaves \qquad \text{(Eq. 11)}$$

Egestion rate (unit g(DW)/day), or the frass production rate, equals total DW of collected faeces for a stage divided by the number of days (Eq. 12). Egestion rate of C and N (g(C or N)/day) were calculated by multiplying the ingestion rate with the C or N content of the faeces (Eq. 13).

$$Egestion\ rate = \frac{total\ faeces\ DW}{no\ of\ days} \qquad \text{(Eq. 12)}$$

$$Egestion\ rate\ of\ C\ or\ N = egestion\ rate \times C\%\ or\ N\%\ in\ faeces \qquad \text{(Eq. 13)}$$

Exuviation rate (unit g(DW)/day) is equal to the molt DW divided by the number of days for the stage before molting (Eq. 14). This was also calculated with regard to C and N (g(C or N)/day) (Eq. 15).

$$Exuviation\ rate = \frac{molt\ DW}{no\ of\ days} \qquad \text{(Eq. 14)}$$

$$Exuviation\ rate\ of\ C\ or\ N = exuviation\ rate \times C\%\ or\ N\ \%\ in\ molt \qquad \text{(Eq. 15)}$$

2.5.2 Approximate digestibility (AD), efficiency of conversion of ingested food (ECI) and efficiency of conversion of digested food (ECD)

The approximate digestibility (AD) is equal to the percentage of ingested food that is assimilated in the insect (Eq. 16) (Scriber & Slansky 1981).

$$AD = 1 - \frac{(egestion\ rate + exuviation\ rate)}{ingestion\ rate} \qquad \text{(Eq. 16)}$$

Biomass gain (unit g/days) is calculated from ratios of larval FW and DW at the start and end of a given stage, divided by the number of days (Eq. 17). The FW:DW ratio was retrieved from Mehdi Cherif, unpublished data.

$$Biomass\ gain = \frac{\left(end\ FW \times \frac{end\ DW}{end\ FW}\right) - \left(start\ FW \times \frac{start\ DW}{start\ FW}\right)}{no\ of\ days} \qquad \text{(Eq. 17)}$$

The growth efficiency, or the efficiency of conversion of ingested food to insect biomass (ECI), is calculated by dividing the biomass gain with the ingestion rate (Eq. 18). For N, ECI should be approximately equal to AD. For DW and C, ECI<AD because of respiration loss of C (Scriber & Slansky 1981).

$$ECI = \frac{biomass\ gain}{ingestion\ rate} \qquad \text{(Eq. 18)}$$

Efficiency of conversion of digested food (ECD) is the biomass gain divided by the difference in ingestion rate when subtracting exuviation and egestion rate (Eq. 19). For N, ECD should be approximately equal to 1, because N is only lost from egestion and exuviation (Scriber & Slansky 1981).

$$ECD = \frac{biomass\ gain}{(ingestion\ rate - exuviation\ rate - egestion\ rate)} \qquad \text{(Eq. 19)}$$

3. Results

3.1 Leaf preservation method

Average initial area for leaves in tubes was 26.04 cm² (± 2.72 S.E.) and for controls 25.65 cm² (± 1.91 S.E.) (five replicates each). After 96 hrs, the average area for leaves in tubes was 25.98 cm² (± 2.71 S.E.) and for controls 19.49 cm² (± 2.04 S.E.) (Figure 11). This was equal to an average decline of 0.23% for leaves in tubes, and 24.64% decline for control leaves. Decline in area after 96 hrs was significantly different between leaves in tubes and control leaves (t-test, p<0.0001). Paired t-test showed that there were significant differences in area in both tubes (p=0.030 and controls (p=0.00084) at 0 hrs and 96 hrs respectively.
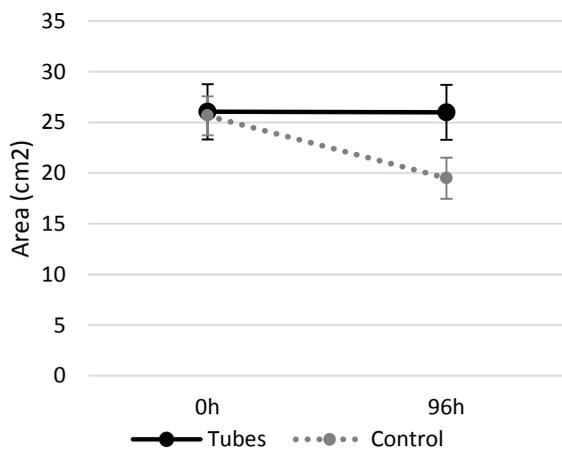

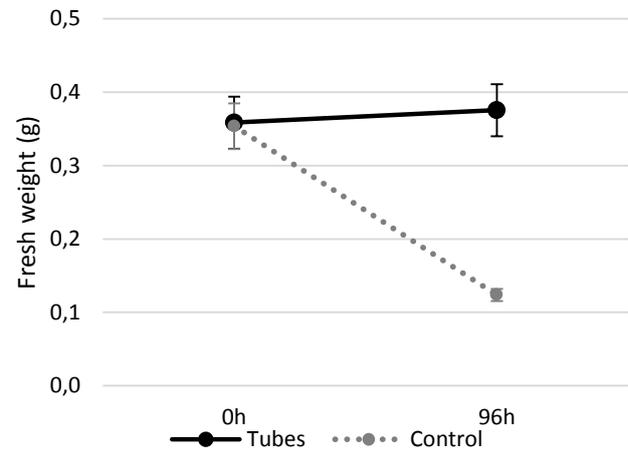Figure 11. Average area (cm²) with S.E. for leaves in tubes and control leaves at 0 hrs and 96 hrs.

Figure 12. Average fresh weight (g) with S.E. for leaves in tubes and control leaves at 0 hrs and 96 hrs.

Average initial fresh weight (FW) for leaves in tubes was 0.358 g (± 0.035 S.E.) and for controls 0.354 g (± 0.031 S.E.). After 96 hrs, the average FW for leaves in tubes was 0.375 g (± 0.035 S.E.) and for controls 0.124 g (± 0.008 S.E.) (Figure 12). This resulted in an average FW increase of 4.94% for leaves in tubes, and an average decrease of 64.68% for control leaves. Difference in FW after 96 hrs between control leaves and leaves in tubes was significant (t-test, p<0.0001). Paired t-test showed that there were significant differences in

FW in both tubes (p=0.068) and controls (p=0.00062) between 0 hrs and 96 hrs. There were no statistical difference between tubes and control leaves in initial FW (p=0.91) and initial area (p=0.92).

3.2 C:N ratio in painted and unpainted leaves
Average C:N and C and N content were analyzed in control leaves, leaves painted with sucrose solution and leaves painted with acetone only (Figure 13 and Table 1).
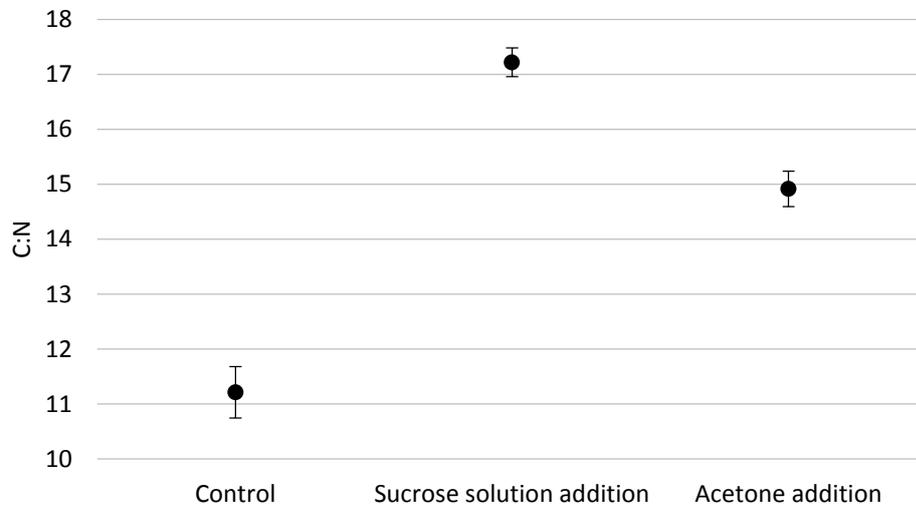

Figure 13. Average C:N ratio with 95% confidence interval in control leaves, leaves painted with sucrose solution and leaves painted with acetone only.

Table 1. Average C and N content as percentage of weight with S.E. in control leaves, leaves with added sucrose solution and leaves with added acetone.

Leaf treatment	C% weight	N% weight
Control	40.69±0.27	3.69±0.17
Sucrose solution addition	40.64±1.23	2.34±0.08
Acetone addition	43.58±1.98	2.81±0.15

A significant difference in C:N were found (ANOVA, p<0.0001) between the three leaf treatments. No significant difference was found between the three leaf treatments when considering C content (ANOVA, p=0.92), nor when comparing control leaves and sucrose solution only (t-test, p=0.97). When considering N content, a significant difference was found between the three treatments (ANOVA, p=0.05), and also between the control and the sucrose solution (t-test, p=0.003).

3.3 Length of developmental stage
The average length of the egg stage was 5.54±0.14 days, the L1 stage was 5.50±0.54 days, the L2 was 4.75±0.65 days, the L3 was 6.44±0.38 days, the prepupa was 2.22±0.15 days and the pupa stage was 6.57±0.20 days (Figure 14).
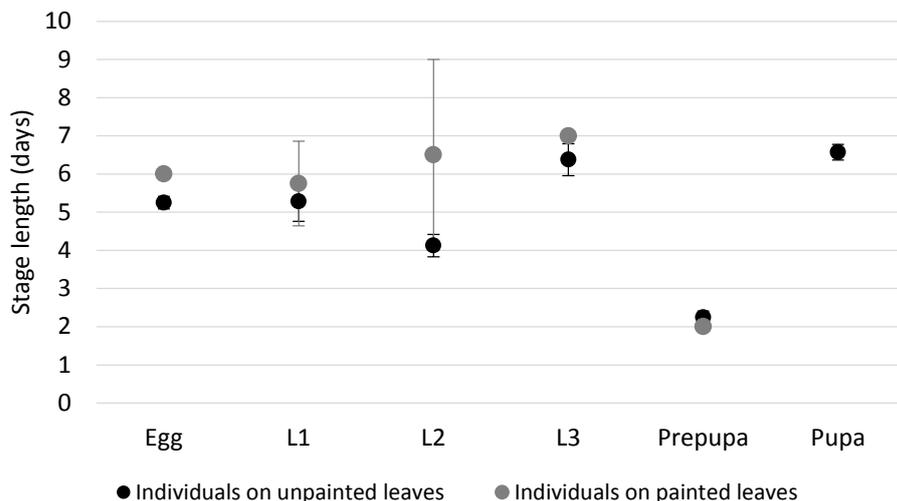
Figure 14. Average stage length with error bars showing S.E. of the developmental stages egg, 1st instar larvae (L1), 2nd instar larvae (L2), 3rd instar larvae (L3), prepupa and pupa in days for individuals on unpainted leaves compared to individuals on painted leaves.

When considering leaf treatment method, no statistical analysis could be made on stage length with difference in C:N ratio because samples were too few. However, one individual with added sucrose showed a longer developmental time for stage L1 (9 days) and L2 (9 days) compared to other individuals.

3.4 Rates of ingestion, egestion and exuviation

Average values of carbon ingestion, egestion and exuviation rates with S.E. for each developmental larval stage was calculated on leaves painted with sucrose and unpainted control leaves. The same was done with nitrogen ingestion, egestion and exuviation rates. When considering ingestion rate, both C and N rates appear to be higher for individuals on painted leaves compared to individuals on unpainted leaves (Figure 15 and 16).
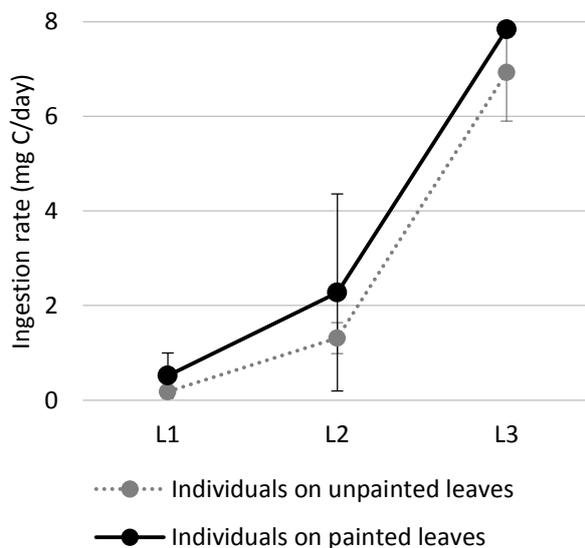

Figure 15. Carbon ingestion rate (mg C/day) with S.E. when possible.
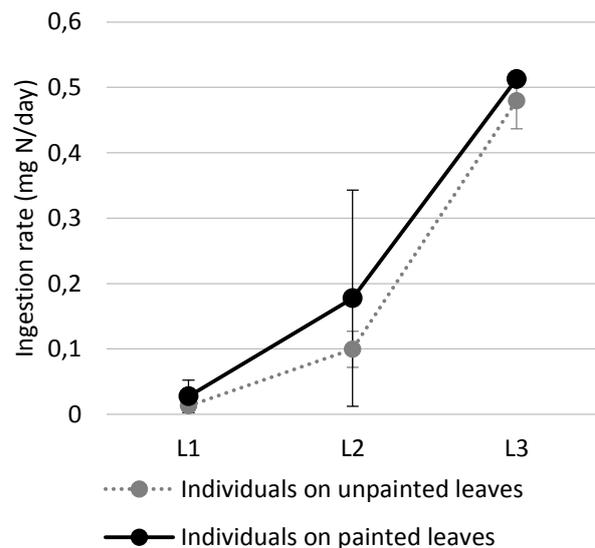

Figure 16. Nitrogen ingestion rate (mg N/day) with S.E. when possible.

For C and N egestion rates, individuals on unpainted control leaves showed a trend of higher rates compared to individuals on leaves painted with sucrose (Figure 17 and 18). For L3, no S.E. is shown due to only one sample.
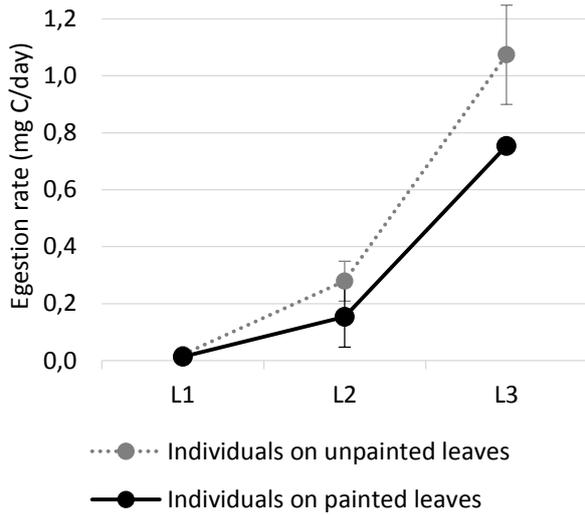
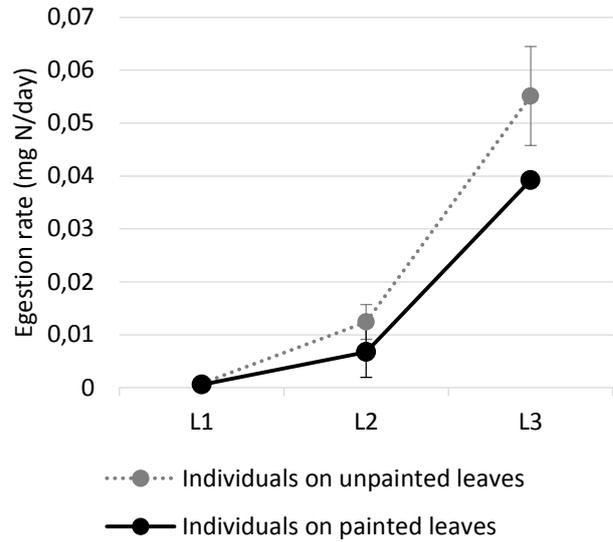Figure 17. Carbon egestion rate (mg C/day) with S.E. when possible.

Figure 18. Nitrogen egestion rate (mg N/day) with S.E. when possible.

C and N exuviation rates appeared to be higher in individuals on unpainted leaves compared to individuals on painted leaves for larvae stage 1 and 2, no data for painted individuals in L3 was obtained (Figure 19 and 20).
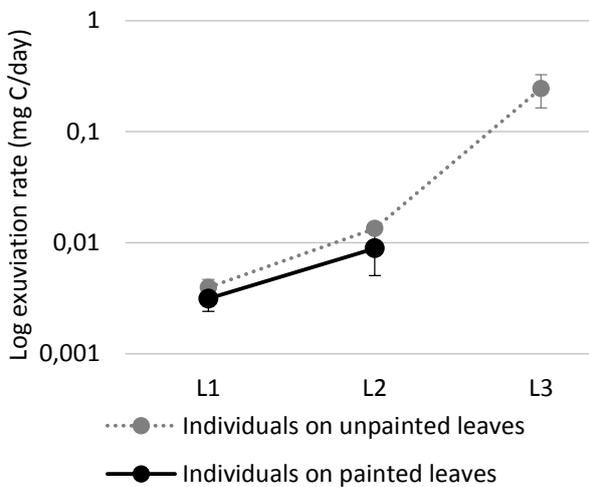

Figure 19. Carbon exuviation rate (mg C/day) with S.E. when possible. L3 stage for individuals on painted leaves is missing.
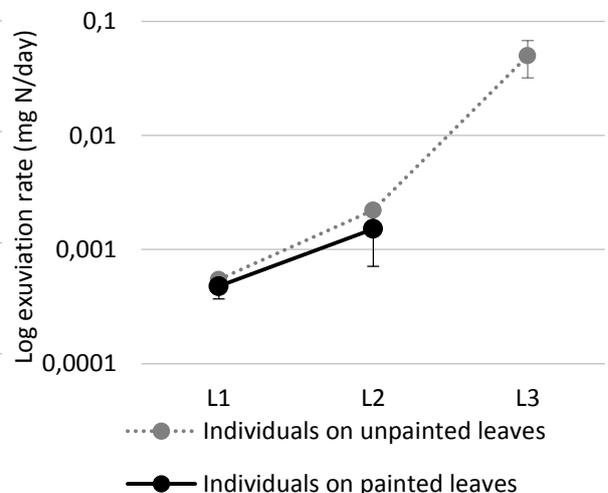

Figure 20. Nitrogen exuviation rate (mg N/day) with S.E. when possible. L3 stage for individuals on painted leaves is missing.

3.5 Approximate digestibility (AD), efficiency of conversion of ingested food (ECI) and efficiency of conversion of digested food (ECD)

Approximate digestibility (AD) was calculated from average values of C and N egestion, ingestion and exuviation rates. AD appears to decrease with higher developmental stage for all samples except C AD between 2nd and 3rd instar larvae for individuals on unpainted leaves (Figure 21).
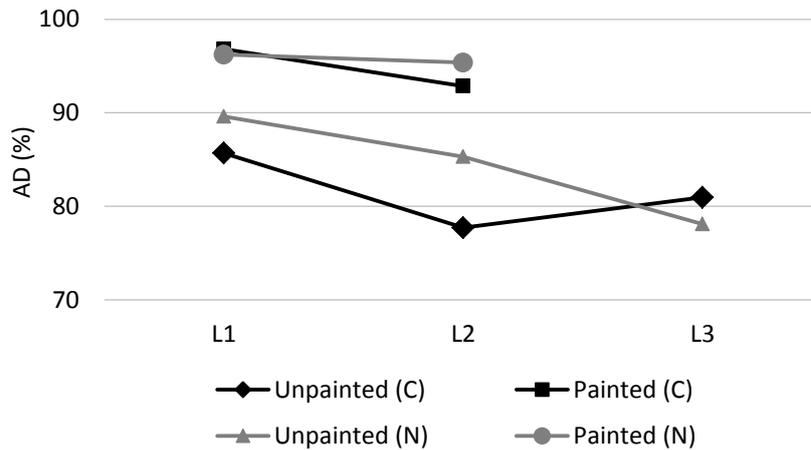
Figure 21. Average C and N approximate digestibility (AD) for individuals on unpainted and painted leaves at the three larvae stages. No samples of L3 on painted leaves.

DW/FW ratio calculated from data (Mehdi Cherif, unpublished) gave for 1st instar an average value of 29.55±7.57%, 2nd instar larvae 23.89±0.47%, 3rd instar larvae 22.47±0.39% and prepupa 25.62±0.34%. There were significant differences between the stage ratios (ANOVA, p=0.02). The ratios for each stage were then multiplied with FW of this study to receive DW values. This was then used to calculate biomass gain which was multiplied with the average C and N content of the insects at each stage (extreme values were excluded) (Mehdi Cherif, unpublished) (Table 2).

Table 2. Biomass gain (mg C or N/day) for the three different stages.

Nutrient	L1	L2	L3
C	0.070	0.43	0.65
N	0.016	0.084	0.12

Biomass gain was then used to get the efficiency of conversion of ingested food (ECI) (Figure 22) and efficiency of conversion of digested food (ECD) (Figure 23).

Figure 22. Efficiency of conversion of ingested food (ECI) for C and N on unpainted leaves for the three different larval stages.
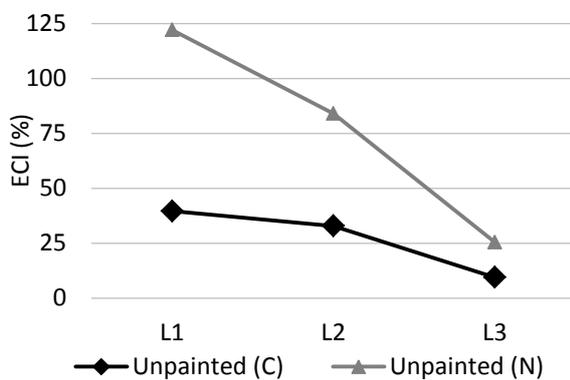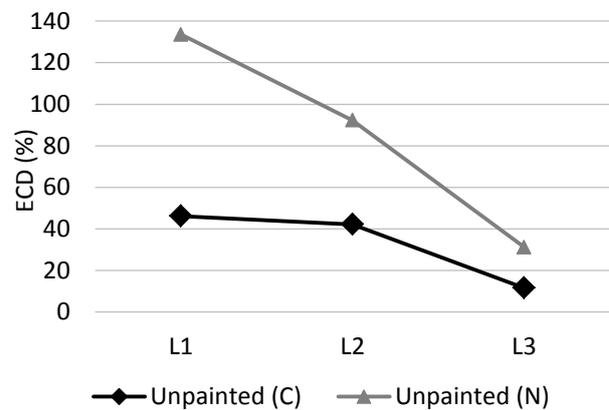

Figure 23. Efficiency of conversion of digested food (ECD) for C and N on unpainted leaves during the three different larval stages.

4. Discussion

There was significant difference in the C:N between control leaves and leaves painted with sucrose solution and acetone (ANOVA, p<0.0001). The painting method must therefore be

considered successful in increasing the C:N ratio. However, the solution did not increase the C content (40.64±1.23%C in painted leaves and 40.69±0.27%C in control leaves, t-test, p=0.97). It rather decreased the N content significantly (2.34±0.08%N in painted leaves compared with 3.69±0.17%N in control leaves, t-test, p=0.003) hence the increased C:N. This result is what to expect when adding C in the form of sucrose, which also contains hydrogen and oxygen ($C_{12}H_{22}O_{11}$) that adds to the weight. When adding sucrose, the whole DW increases in proportion and the C% remains approximately the same. An increase in C as % of DW is only likely when C is added in the form of highly saturated lipids (($CH_2)_n$). This test only included five replicates each, which one can argue is too few to exclude the possibility of obtaining the results due to chance. To be certain about this, more replicates would have been favorable.

Another issue was that the sucrose solution became sticky when put in the climate room so any larvae feeding on the lower side became glued to it and eventually died. Five eggs were put on painted leaves, all hatched but only three individuals survived to L2. Two of those survived to L3 and finally one survived that stage and pupated, hence 1 out of 5 made it to the adult stage. The survivorship among individuals on unpainted leaves was much better with 9 individuals making it to adult stage out of 14. It is possible that the leaves never truly absorbed the solution. Perhaps the added sucrose solution led to an osmotic outflow of water from the leaf cells. My personal observation is that the leaves with painted sucrose needed more water to be refilled in the Eppendorf tubes than unpainted leaves. To summarize, the larvae could not feed on the painted leaves in the same manner as on the unpainted leaves. When considering that, the method did not work well. Perhaps a solution with another carbohydrate would have been better, but sucrose was chosen since it was the main carbohydrate form in aspen leaves. The painting method had successfully been tried by Noseworthy and Despland (2006), who supplemented leaves of trembling aspen (*Populus tremuloides*) with sucrose and casein. However, the sucrose concentration in their trial was never stated and perhaps this is the reason to our different outcomes. A lower concentration than the one used in this study is perhaps better.

Regarding length of developmental stage, it is hard to estimate what is caused by the leaf treatment. One individual on painted leaves showed longer developmental time, but since there were so few surviving replicates, no statistics could be made. However, this supports the hypothesis that increased C to N would lengthen the development. Also, there were differences in ingestion, egestion and exuviation rates between the individuals on painted and unpainted leaves. Whether these changes are significant or due to chance cannot be said because of too few replicates on the painted leaves, but the trend does imply that there could be a difference that might be interesting to investigate further. The ingestion rate is higher among individuals on painted leaves, which could imply that these individuals need to eat more in order to retrieve the N needed. However, the egestion rate is lower among individuals on painted leaves compared to individuals on unpainted leaves, which contradicts the theory that insects feeding on leaves with higher C:N would egest more when trying to get rid of the C excess. This might come from the type of carbohydrate added, sucrose may show high assimilation rates by insects which then would lower the egestion rate. The exuviation rate is lower among individuals on painted leaves, which is what to expect from longer developmental time.

When calculating the ingestion rates for the different stages some rates received negative values and these were excluded from the calculations. This originated in that some leaves had higher DW at the end after been fed on compared to the estimated initial DW. The initial DW was estimated from the ratio of end DW/area multiplied with the initial area and the negative results could be explained by the measurements of the eaten area in the leaf area meter. The difference in leaf area before and after being fed on was in some cases negative, inconsistent and not accurate enough. Also, some leaves were fed on so that parts of it had fallen off and desiccated. This meant that it was counted as eaten area even if it wasn't. Perhaps another

method in measuring the area would have been better, one discussed would have been to use a transparent paper with mm-grid.

The accuracy in egestion rates for L1 can also be disputed; the faeces were very small and existed only in very small amounts. It was hard to retrieve all of it and some were lost during handling, freezing and weighing.

The method of putting leaves in Eppendorf tubes with water resulted in a significant decline in area of about 0.23% (paired t-test, p=0.030) and an increase in FW of about 4.94%, although not significant (paired t-test, p=0.068). Preferably, the leaf area should not have differed significantly between 0 and 96 hrs, but the uncertainty of the accuracy of the leaf area meter should be taken into account and the decrease is also a very small one.

Another aspect that ought to be discussed is the leaf quality. The leaves chosen from the 12 individuals of *P. tremula* x *tremuloides* (T89) had to be within the size of the petri dishes (120x120 mm) and older leaves were preferred. The leaves were picked during a period of 6 weeks, which means that the older leaves in the beginning of that period were younger than the older leaves in the end. Also, phenolic compounds in leaves are known to increase when exposed to herbivory, therefore the leaf quality might be lower in the leaves picked later during the period. When testing the C and N content of 10 control leaves, the C content ranged from 39.61-42.22% and the N content 3.00-4.59%. However, all those leaves were picked at one occasion. A possible difference in leaf quality might explain why one adult individual ate nothing on its leaf for 14 days during the feeding trial.

When comparing differences between the three larval stages, AD decreases with higher developmental stage in all cases except between L2 and L3 on unpainted leaves (Figure 22). This decrease could be due to less selective feeding by L2 and L3 compared to L1, which results in increased consumption of less digestible fiber. According to Scriber and Slansky (1981) this is often the case with leaf chewing insects. The ECD and ECI also decreased from L1 through L3 (Figure 23 and 24), however, ECD usually tends to increase while ECI may decrease, increase or show little change (Scribed and Slansky 1981). The biomass gain for individuals on painted leaves could not be calculated since the C and N content of the insects were retrieved from data from another study (Mehdi Cherif, unpublished). Hence nothing can be said about the ECI and ECD among these individuals. Individuals on unpainted leaves received 122.09% for nitrogen ECI and 133.54% for nitrogen ECD. However, efficiency values above 100% are impossible. The values might perhaps be explained by the ingestion rate which could contain wrong estimates of ingested DW. It is also possible that the inclusion of data from another study when calculating different rates might be misleading.

To conclude, a significant increase in C:N was successfully achieved, but the sucrose painting method still did not meet the wanted result. For future experiments, another method in increasing the carbohydrate content should be tested. AD, ECD and ECI all increased from 1st, through 2nd and 3rd instar larvae with generally higher efficiencies for nitrogen than carbon. In order to answer the question whether that would affect the nutrient cycling and composition in a community, a larger and more thorough study needs to be done.

5. Acknowledgement

6. References

Augustin, S., Courtin, C. & Delplanque, A. 1993. Poplar clones effect on development, mortality, and fecundity of *Chrysomela* (= *Melasoma*) *populi*) L. and *Chrysomela tremulae* F. (*Col., Chrysomelidae*). *Journal of Applied Entomology*. 116(1-5): 39–49. doi:10.1111/j.1439-0418.1993.tb01166.x

Augustin, S. & Lévieux, J. 1993. Life history of the poplar beetle *Chrysomela tremulae* F. in the central region of France. *The Canadian Entomologist*. 125(2): 399-401. doi:10.4039/Ent125399-2

Behmer, S.T. 2009. Insect Herbivore Nutrient Regulation. *Annual Review of Entomology*. 54: 165-187. DOI:10.1146/annurev.ento.54.110807.090537

Berner, D., Blanckenhorn, W. U., & Körner, C. 2005. Grasshoppers cope with low host plant quality by compensatory feeding and food selection: N limitation challenged. *Oikos*. 111(3):525–533. doi:10.1111/j.1600-0706.2005.14144.x

Bradford, M.A., Gancos, T. & Frost C.J. 2008. Slow-cycle effects of foliar herbivory alter the nitrogen acquisition and population size of Collembola. *Soil Biology & Biochemistry*. 40(5): 1253-1258. DOI:10.1016/j.soilbio.2007.12.001

Chow, P.S. & Landhausser, S.M. 2004. A method for routine measurements of total sugar and starch content in woody plant tissues. *Tree Physiology*. 24(10): 1129 –1136. doi: 10.1093/treephys/24.10.1129

Findlay, S., Carreiro, M., Krischik, V. & Jones, C.G. 1996. Effects of damage to living plants on leaf litter quality. *Ecological Applications*. 6(1): 269-275. doi:10.2307/2269570

Fogal, ,W.H. & Slansky, Jr. F. 1985. Contribution of feeding by European pine sawfly larvae to litter production and element flux in Scots pine plantations. *Canadian Journal of Forest Research*. 15(3): 484-487, doi:10.1139/x85-079

Frost, C.J. & Hunter, M.D. 2004. Insect canopy herbivory and frass deposition affect soil nutrient dynamics and export in oak mesocosms. *Ecology*. 85(12): 3335-3347. DOI:10.1890/04-0003

Gruppe, A., Fußeder, M. & Schopf, R. 1999. Short rotation plantations of aspen and balsam poplar on former arable land in Germany: defoliating insects and leaf constituents. *Forest Ecology and Management*. 121(1–2): 113–122. doi:10.1016/S0378-1127(98)00560-X

Hemming, D.C. & Lindroth, R.L. 2000. Effects of phenolic glycosides and protein on gypsy moth (*Lepidoptera: Lymantriidae*) and forest tent caterpillar (*Lepidoptera: Lasiocampidae*) performance and detoxication activities. *Environmental Entomology*. 29(6): 1108-1115. doi:10.1603/0046-225X-29.6.1108

Hollinger, D.Y. 1986. Herbivory and the cycling of nitrogen and phosphorus in isolated California oak trees. *Oecologia*. 70(2): 291–297. doi:10.1007/BF00379254

Hunter, M.D. 2001. Insect population dynamics meets ecosystem ecology: effects of herbivory on soil nutrient dynamics. *Agricultural and Forest Entomology*. 3(2): 77-84. doi:10.1046/j.1461-9563.2001.00100.x

Jolivet, P., Petitpierre, E. & Hsiao, T.H. 1988. *Biology of Chrysomelidae*. Series Entomologica. Springer Netherlands. ISBN:978-94-010-7896-2

Leplé, J.C., Bonadé-Bottino, M., Augustin, S., Pilate, G., Lê Tân, V.D., Delplanque, A., Cornu, D. & Jouanin, L. 1995. Toxicity to *Chrysomela tremulae* (*Coleoptera: Chrysomelidae*) of transgenic poplars expressing a cysteine proteinase inhibitor. *Molecular Breeding*. 1(4): 319-328. doi:10.1007/BF01248409

Lovett, G.M. & Ruesink, A.E. 1995. Carbon and nitrogen mineralization from decomposing gypsy moth frass. *Oecologia*. 104(2): 133-138. doi:10.1007/BF00328577

McNaughton, S.J., Ruess, R.W. & Seagle, S.W. 1988. Large Mammals and Process Dynamics in African Ecosystems. BioScience. 38(11): 794-800. doi:10.2307/1310789

Madritch, M.D., Donaldson, J.R., & Lindroth, R.L. 2007. Canopy herbivory can mediate the influence of plant genotype on soil processes through frass deposition. *Soil Biology & Biochemistry*. 39(5): 1192–1201. doi:10.1016/j.soilbio.2006.12.027

Mandre, M., Tullus, A., Klõšeiko, J., Lukjanova, A. & Tullus, H. 2011. Variation of carbohydrates and lignin in hybrid aspen (*Populus tremula* x *P. tremuloides*) on alkaline soil. *Cellulose Chemistry and Technology*. 45(5-6): 299-311.

Mattson, W.J. & Addy, N.D. 1975. Phytophagous insects as regulators of forest primary production. *Science*. 190(4214):515-522. doi:10.1126/science.190.4214.515

Mulder, C.P.H. 1999. Vertebrate herbivores and plants in the Arctic and subarctic: effects on individuals, populations, communities and ecosystems. *Perspectives in Plant Ecology, Evolution and Systematics*. 2(1): 29-55. doi:10.1078/1433-8319-00064

Noseworthy, M.K. & Despland, E. 2006. How do primary nutrients affect the performance and preference of forest tent caterpillars on trembling aspen? *The Canadian Entomologist*. 138(3): 367-375. doi:10.4039/n05-076

Pastor, J. & Naiman, R.J. 1992. Selective Foraging and Ecosystem Processes in Boreal Forests. *The American Naturalist*. 139(4):690-705. doi:10.1086/285353

Salminen, J.P. & Lempa, K. 2002. Effects of hydrolysable tannins on a herbivorous insect: fate of individual tannins in insect digestive tract. *Chemoecology*. 14(4): 203-211. doi:10.1007/PL00012670

Schowalter, T.D., Hargrove, W.W. & Crossley, D.A. Jr. 1986. Herbivory in forested ecosystems. *Annual Review of Entomology*. 31: 177-196. doi:10.1146/annurev.en.31.010186.001141

Scriber, J. M. 1977. Limiting effects of low leaf-water content on the nitrogen-utilization, energy budget, and larval growth of *Hyalophora cecropia* (Lepidoptera, Saturniidae). *Oecologia*. 28(3):269–287. doi:10.1007/BF00751605

Scriber, J. M., & Slansky, F. (1981). The nutritional ecology of immature insects. *Annual Review of Entomology*. 26: 183-211. doi:10.1146/annurev.en.26.010181.001151

Seastedt, T.R. & Crossley, D.A. 1984. The Influence of Arthropods on Ecosystems. *Bioscience*. 34(3): 157-161. doi:10.2307/1309750

Tukey, H.B. & Morgan, J.V. 1963. Injury to foliage and its effects upon the leaching of nutrients from above-ground plant parts. *Physiologia Planta*. 16: 557-564. doi:10.1111/j.1399-3054.1963.tb08333.x

Dept. of Ecology and Environmental Science (EMG)
S-901 87 Umeå, Sweden
Telephone +46 90 786 50 00
Text telephone +46 90 786 59 00
www.umu.se