307.qxd 7/25/05 7:29 PM Page 153

Chemoecology 15:153–159 (2005)
0937–7409/05/030153–7
© Birkhäuser Verlag, Basel, 2005
DOI 10.1007/s00049-005-0307-7

CHEMOECOLOGY

Biochemical transformation of birch leaf phenolics in larvae of six species of sawflies

Maria Lahtinen[1], Lauri Kapari[2], Vladimir Ossipov[1,2], Juha-Pekka Salminen[1], Erkki Haukioja[2] and Kalevi Pihlaja[1]

[1]Laboratory of Organic Chemistry and Chemical Biology, Department of Chemistry, University of Turku, FI-20014 Turku, Finland
[2]Section of Ecology, Department of Biology, University of Turku, FI-20014 Turku, Finland

Summary. We investigated the biochemical transformation of individual phenolic compounds of mountain birch leaves in larvae of six birch-feeding sawfly species: *Amauronematus amplus, Pristiphora alpestris, Nematus brevivalvis, Priophorus pallipes, Arge* sp. and *Nematus viridis* by comparing the phenolic residues in larval faeces to those of their leaf diet. Partial hydrolysis of hydrolysable tannins, isomerisation of chlorogenic acid and glycosylation of flavonoid aglycones were observed in all studied species. Moreover, we found considerable among-species variation in the composition of phenolic compounds in larval faeces. In addition to foliar phenolics, seventeen non-foliar phenolic metabolites, including eight phenolic acids and nine flavonoid glycosides were detected from the faeces. Of the non-foliar phenolic acids, four were egested species-specifically and only two by all six sawfly species. We also detected differences in the ratios of chlorogenic acid isomers in the faeces of different species, which can indicate different physiological conditions in the guts of studied larvae. In addition to the qualitative differences, quantitative differences were detected in the egestion of chlorogenic acids, possible *o*-quinone precursors in the larvae. Detected differences, either qualitative or quantitative, could not be explained by seasonal changes in the content of compounds in the leaf diet.

Key words. Birch – sawfly larvae – biochemical transformation – phenolic compounds

Introduction

Birch leaves contain a wide variety of phenolic compounds, such as flavonoids, tannins and phenolic acids, which can affect consumption rates and pupal masses of herbivores consuming the leaves (e.g. Haukioja 2003). Salminen *et al.* (2004) proposed that understanding the true effects of particular phenolic compounds on insect performance requires knowledge of how phenolics are metabolized in the digestive tract of insects. Recently, we have reported fates of individual birch (*Betula pubescens*) leaf phenolics in larvae of the geometrid moth *Epirrita autumnata*, the main defoliator of birch (Salminen & Lempa 2002; Salminen *et al.* 2004). However, so far we have not studied the fate of birch leaf phenolics in any other insect species, although we know that birch is also attacked, for example, by numerous species of sawflies; mountain birch alone accommodates close to 40 sawfly species, most of which are birch specialists (Hanhimäki *et al.* 1995). In the present study, we expand our compound-specific birch-herbivore studies to six species of birch-feeding sawflies: *Amauronematus amplus, Pristiphora alpestris, Nematus brevivalvis, Priophorus pallipes, Arge* sp. and *Nematus viridis*.

In our earlier studies with birch and flush-feeding *E. autumnata* we have reported particular reactions of foliar phenolics in larvae, such as partial hydrolysis of hydrolysable tannins, isomerisation of chlorogenic acid and glycosylation of flavonoid aglycones (Salminen & Lempa 2002; Salminen *et al.* 2004). Because of drastic changes in the composition of foliar phenolics during the growing season (e.g. Salminen *et al.* 2001; Riipi *et al.* 2002; Haukioja 2003; Valkama *et al.* 2004), we supposed that sawflies, which mostly consume mature leaves (Hanhimäki *et al.* 1995; Martel *et al.* 2001), may have developed different ways for metabolizing phenolics compared to flush feeding *E. autumnata*. Lahtinen *et al.* (2004) showed that high levels of flavonoid aglycones on the surface of young birch leaves can be harmful for first instar *E. autumnata* larvae and Salminen *et al.* (2004) showed that fifth instar *E. autumnata* larvae are able to detoxify aglycones by glycosylating them. Since the flavonoid aglycone content of birch leaves declines rapidly as leaves mature (Valkama *et al.* 2004) and sawflies tend to consume these aglycone poor leaves, we might expect that the ability to detoxify flavonoid aglycones is more a property of *E. autumnata* than of sawfly larvae. There is large seasonal variation also among the sawfly species in the maturity of the leaves they feed on, the timing of the last larval instar ranging from early July to early September. Thus, differences in phenology might correspond to varying metabolic fates of phenolics even among the sawflies. Species- or even instar-specific ways to metabolize ingested leaf material could be assumed, because Martel *et al.* (2001) found that birch sawflies were poor in using leaves that were phenologically earlier or later in development than the leaves that they typically encounter.

Correspondence to: Maria Lahtinen, e-mail: makahe@utu.fi

307.qxd 7/25/05 7:29 PM Page 154

To test hypotheses mentioned above, the aims of the present study were to document the biochemical transformation of individual birch leaf phenolic compounds in larvae of six sawfly species by comparing the phenolic compounds in larval faeces to those of their leaf diet and to examine possible differences in phenolic metabolism between species. The factors behind the detected differences are also discussed.

2. Materials and methods

2.1. Experimental design

The experiments were conducted at the Kevo Subarctic Research Station in Utsjoki, Northern Finland (69°45'N, 27°00'E) during the summer (June-August) of 2001. Of the six sawfly species tested (*Amauronematus amplus* Konow, *Pristiphora alpestris* Konow, *Nematus brevivalvis* Thomson, *Priophorus pallipes* Lepeletier, *Arge* sp. Schrank and *Nematus viridis* Stephens), *Arge* sp. belongs to Argidae, the others to Tenthredinidae (subfamily Nematinae). The original experimental design included 14 trees with three shaded branches and three control branches in each tree. Only the data from control branches is presented here. Of the three branches per tree branch A was used for *A. amplus* and *P. pallipes*, branch B for *N. brevivalvis* and *N. viridis* and branch C for *P. alpestris* and *Arge*. We have previously demonstrated that, unlike in primary metabolites, within-tree variation in foliar phenolics is low compared to among-tree variation (Suomela *et al.* 1995).

We tried to test all the species during the early days of the ultimate instar except *N. brevivalvis* which was tested in the penultimate instar. Due to variation in the number of instars and large variability within broods, some penultimate instar larvae were included in the tests with other species (most substantially in *N. viridis*). *A. amplus* was tested on July 6, *P. alpestris* on July 10, *N. brevivalvis* on July 13, *P. pallipes* on July 20, *Arge* sp. on July 26, and *N. viridis* on August 15. The tested larvae were haploid males (produced by unmated females), except in the experiment with *P. alpestris* which included both males and females. The emerging adult sawfly females were allowed to oviposit on foliage enclosed in mesh bags, and the larvae were allowed to grow on these branches until taken into laboratory approximately one week before the experiments. The larvae were thus reared throughout the study on mountain birch leaves, but during the early development the trees were not the same for different species or broods within species. Approximately 18 hours before, and during the experiments, the larvae of all species were fed in the lab on leaves from the same 14 individual trees. Five (four for *P. pallipes*) larvae belonging to different broods were tested in each tree. The experiments were conducted in growth chambers at 13 ± 1 °C and with a 24 hour light cycle (typical for our high latitude study site).

Faeces samples were collected from the rearing vials once during the 24 h experiment, which might have enabled post-intestinal modification of the larval metabolites and the formation of artefacts in the faeces. However, earlier Salminen *et al.* (2004) showed that the only HPLC-detectable difference between fresh and 24 h old faeces of *E. autumnata*, was the appearance of uric acid in the latter types of samples. On the basis of this study, we have assumed that also in the faeces of sawfly larvae the phenolic composition stays relative constant during the 24 h experiment. The formation of uric acid was also detected from the faeces of sawfly larvae.

2.2. Sample preparation

Freeze-dried faeces from five (four for *P. pallipes*) larvae per tree were pooled for tree-specific samples before the chemical analyses. Preparation of leaf and faeces samples for HPLC-DAD/HPLC-ESI-MS analyses of their vacuolar phenolics were conducted as previously described (Salminen *et al.* 1999, 2001; Salminen & Lempa 2002). Six leaves from each branch were collected for analyses, in which the contents of individual phenolic compounds (hydrolysable tannins, catechin, chlorogenic acids, coumaroylquinic acids, and flavonoid glycosides) were determined. Flavonoid aglycones were extracted with 95 % ethanol from two leaves of each branch as in Valkama *et al.* 2003. The sampling dates of leaves were July 6, 13, 26 and August 16.

2.3. HPLC-DAD and HPLC–ESI-MS

Leaf and faeces extracts were analysed with HPLC-DAD at 280 nm, 315 nm and 349 nm. The HPLC system (Merck-Hitachi, Tokyo, Japan) consisted of a pump L-7100, a diode array detector L-7455, a programmable autosampler L-7250, and an interface D-7000. Column and chromatographic conditions were as described earlier (Salminen *et al.* 1999), except that 0.1M H_3PO_4 was replaced with 0.05M H_3PO_4. A selected set of samples was analysed also with HPLC-ESI-MS as in Salminen *et al.* (1999) except that the datasystem used was Analyst Software 1.1.

2.4. Compound identification and quantification

Compounds were identified on the basis of their UV and mass spectra and retention times reported in the literature (Ossipov *et al.* 1995, 1996; Salminen *et al.* 1999, 2001; Valkama *et al.* 2003; Salminen *et al.* 2004) and by comparison to authentic standards (protocatechuic acid from Sigma). Galloylquinic acids were identified by their UV and mass spectra: Their UV spectra were similar to that of gallic acid and mass spectra showed ions at m/z 343, m/z 191 and m/z 169, corresponding to $[M–H]^-$ of galloylquinic acid, $[M–H]^-$ of quinic acid and $[M–H]^-$ of gallic acid.

Galloylglucoses were quantified as monogalloyl glucose, pedunculagin and its derivative as pedungulacin, catechin as catechin, chlorogenic acids (isomers and derivatives) as chlorogenic acid, coumaroylquinic acids as coumaric acid, gallic acid and galloylquinic acids as gallic acid, protocatechuic acid as protocatechuic acid and flavonoid glycosides as corresponding aglycones. Unknown flavonoid glycosides were quantified as quercetins. Quantification was done both in relative (mg/g dry weight) and absolute values (mg per sample). Absolute values were used for the calculation of egestion percentages of compounds. The ingested amounts were calculated based on measurements of dry weight consumption by the larvae during the growth trials. Initial and final leaf areas of the leaves were measured from digital photographs (Olympus C-2040/resolution 640 * 480) using the Sigma Scan Pro 4 (©SPSS Inc.) image analysis program. Partly consumed leaves were vacuum dried and weighted. Dry weight in mg per area from the leaves was used to calculate the initial dry weight of each experimental leaf. Tree specific means for the dry weight consumption and the dry weight of the faeces were used in calculation of egestion percentages of compounds.

2.5. Statistical analyses

Tree-specific values for the amount egested in relation to the amount ingested (%) were used as dependent variables in statistical tests for differences between species in the relative amounts of egested *o*-dihydroxylated compounds (chlorogenic acids and quercetin glycosides) and new non-foliar flavonoid glycosides. The differences between species in egestion percentages were tested with non-parametric Kruskal-Wallis tests using SAS 8.2 statistical software (SAS institute inc. 1999–2001).

3. Results and discussion

Even though the phenolic composition in leaves stayed qualitatively constant during the experiment, the compound composition in larval faeces was found to differ significantly among the sawfly species (Tables 1–3). All species egested non-foliar, previously undetected products of larval phenolic metabolism. Some metabolites were detected in faeces of all species while some were characteristic to one or two species only.

307.qxd 7/25/05 7:29 PM Page 155

Table 1 Quantities of hydrolysable tannins and catechin (mg/g dry weight) present in both leaf and faeces samples. The values shown are means ± SE of n = 14 in both faeces and leaf samples

Hydrolysable tannins and catechin	Leaf diet				Faeces					
	July 6	July 13	July 26	August 16	July 6 *A. amplus*	July 10 *P. alpestris*	July 13 *N. brevivalvis*	July 20 *P. pallipes*	July 26 *Arge* sp.	August 15 *N. viridis*
Monogalloyl glucose	4.70 ± 0.99	3.49 ± 0.81	2.86 ± 0.76	2.97 ± 0.67	–	–	–	–	–	–
Digalloyl glucose	0.91 ± 0.21	1.05 ± 0.21	0.88 ± 0.25	1.26 ± 0.24	–	–	–	–	–	–
Trigalloyl glucose	1.38 ± 0.37	1.40 ± 0.26	1.04 ± 0.27	1.04 ± 0.25	–	–	–	–	–	–
Pedunculagin	2.14 ± 0.67	2.09 ± 0.57	3.25 ± 1.86	1.99 ± 0.61	2.24 ± 0.83	2.30 ± 0.78	1.87 ± 0.50	1.60 ± 0.50	1.82 ± 0.68	1.79 ± 0.61
Pedunculagin derivative	4.05 ± 0.78	3.03 ± 0.62	2.19 ± 0.45	2.76 ± 0.59	–	–	–	–	–	–
Catechin	2.55 ± 0.26	2.48 ± 0.22	2.86 ± 0.32	3.64 ± 0.41	–	–	–	2.35 ± 0.19	–	–

Table 2 Quantities of phenolic acids (mg/g dry weight) present in both leaf and faeces samples. The values shown are means ± SE of n = 14 in both faeces and leaf samples

Phenolic acid	Leaf diet				Faeces					
	July 6	July 13	July 26	August 16	July 6 *A. amplus*	July 10 *P. alpestris*	July 13 *N. brevivalvis*	July 20 *P. pallipes*	July 26 *Arge* sp.	August 15 *N. viridis*
Chlorogenic acid	11.06 ± 1.43	9.76 ± 1.23	8.44 ± 1.29	8.79 ± 1.24	4.39 ± 0.74	4.06 ± 0.83	4.45 ± 0.74	5.68 ± 0.83	5.28 ± 0.73	3.61 ± 0.75
Coumaroylquinic acid	0.49 ± 0.09	0.31 ± 0.06	0.20 ± 0.04	0.15 ± 0.03	1.17 ± 0.10	0.18 ± 0.05	0.50 ± 0.06	0.26 ± 0.04	0.25 ± 0.04	0.26 ± 0.04
Neochlorogenic acid	0.28 ± 0.12	0.29 ± 0.10	0.19 ± 0.09	0.17 ± 0.09	3.60 ± 0.55	1.78 ± 0.39	1.81 ± 0.26	1.18 ± 0.11	1.26 ± 0.16	0.84 ± 0.15
Chlorogenic acid isomer	–	–	–	–	2.85 ± 0.45	1.56 ± 0.33	1.87 ± 0.25	1.45 ± 0.15	1.63 ± 0.21	1.00 ± 0.19
Chlorogenic acid derivative I	–	–	–	–	–	–	–	0.96 ± 0.10	–	–
Chlorogenic acid derivative II	–	–	–	–	–	–	–	2.16 ± 0.24	–	–
Chlorogenic acid derivative III	–	–	–	–	–	–	–	0.94 ± 0.13	–	–
Gallic acid	–	–	–	–	2.45 ± 0.71	1.45 ± 0.27	0.47 ± 0.10	1.00 ± 0.24	0.45 ± 0.11	0.31 ± 0.11
Galloylquinic acid isomer I	–	–	–	–	0.14 ± 0.05	–	0.69 ± 0.17	0.13 ± 0.07	0.83 ± 0.21	0.22 ± 0.08
Galloylquinic acid isomer II	–	–	–	–	–	0.56 ± 0.11	0.40 ± 0.10	–	0.38 ± 0.09	–
Protocatechuic acid	–	–	–	–	–	3.02 ± 0.16	–	–	–	–

3.1. Transformation of hydrolysable tannins

Possible reactions for hydrolysable tannins in an insect's gut are their hydrolysis, oxidation, or adsorption on the peritrophic envelope (Appel & Martin 1990; Appel 1993; Barbehenn & Martin 1994; Barbehenn *et al.* 1996; Salminen & Lempa 2002). With the sawfly larvae studied here, the transformation of hydrolysable tannins was similar among the species; of the foliar tannins only pedunculagin could be detected in the faeces (see Table 1). Although the leaf diet contained more than 5.7 mg/g (seasonal average) of galloylglucoses, they were not found in the faeces as such. Instead, 0.3-2.5 mg/g of gallic acid, the hydrolysis product of galloylglucoses, was detected in the faeces of all species, reflecting at least partial hydrolysis of these tannins in studied larvae. Interestingly, although pedunculagin was egested at different levels (1.6-2.3 mg/g), the detected pedunculagin derivative was totally retained, oxidized or degraded to undetectable catabolites in the digestive tract of all larvae. Salminen and Lempa (2002) showed that individual galloylglucoses differ in their fates within the gut of *E. autumnata* larvae and here we observed the same to be true with individual ellagitannins (pedunculagin and its derivative) in the guts of six species of sawfly larvae. Different fates of individual tannins in larvae might reflect their variable biological activities. In addition to pedunculagin, *P. pallipes* also produced catechin to the faeces, while the other species did not.

There are several factors affecting the biological activity of hydrolysable tannins in plant-herbivore interactions. Tannin oxidation products, such as quinones, and oxidation by-products including different reactive oxygen species can cause enzyme inactivation, membrane lipid peroxidation, and strand breaks in DNA (Appel 1993) Reactive *o*-quinones can also form covalent bonds to nucleophilic group of amino acids decreasing their assimilation in herbivore digestive tract (Felton *et al.* 1989). The ability of tannins as such to precipitate proteins has been suggested as an important factor reducing the suitability

307.qxd 7/25/05 7:29 PM Page 156

Table 3 Quantities of flavonoids (mg/g dry weight) present in both leaf and faeces samples. The values shown are means ± SE of n = 14 in both faeces and leaf samples

	Leaf diet				Faeces					
					July 6	July 10	July 13	July 20	July 26	August 15
Flavonoids	July 6	July 13	July 26	August 16	*A. amplus*	*P. alpestris*	*N. brevivalvis*	*P. pallipes*	*Arge* sp.	*N. viridis*
Flavonoid aglycones[a]	5.16 ± 0.47	4.49 ± 0.50	3.33 ± 0.23	3.48 ± 0.28	–	–	–	–	–	–
Kaempferol-3-*O*-glucoside	0.39 ± 0.06	0.42 ± 0.05	0.36 ± 0.03	0.43 ± 0.05	0.95 ± 0.14	0.96 ± 0.16	0.99 ± 0.16	1.13 ± 0.16	0.71 ± 0.08	1.07 ± 0.16
Kaempferol-3-*O*-rhamnoside	0.60 ± 0.15	0.52 ± 0.12	0.39 ± 0.07	0.40 ± 0.05	–	–	–	–	–	–
Kaempferol glycoside I	0.91 ± 0.14	0.88 ± 0.13	0.73 ± 0.11	0.77 ± 0.13	1.24 ± 0.13	1.42 ± 0.14	1.26 ± 0.15	1.44 ± 0.14	1.43 ± 0.13	1.32 ± 0.14
Kaempferol glycoside II	0.19 ± 0.10	0.12 ± 0.01	0.11 ± 0.01	0.17 ± 0.01	–	0.23 ± 0.04	–	0.28 ± 0.04	0.68 ± 0.13	0.11 ± 0.01
Kaempferol glycoside III	0.12 ± 0.05	0.12 ± 0.06	0.11 ± 0.04	0.16 ± 0.06	–	–	–	–	–	–
Myricetin-3-*O*-galactoside	2.03 ± 0.24	1.81 ± 0.25	1.62 ± 0.26	1.70 ± 0.27	1.68 ± 0.23	1.88 ± 0.28	1.16 ± 0.19	2.03 ± 0.28	1.66 ± 0.24	1.11 ± 0.24
Myricetin-3-*O*-glucuronoside	0.57 ± 0.12	0.44 ± 0.07	0.38 ± 0.08	0.35 ± 0.08	0.38 ± 0.08	0.51 ± 0.12	0.27 ± 0.07	0.42 ± 0.08	0.33 ± 0.06	0.24 ± 0.06
Myricetin glycoside	0.22 ± 0.11	0.12 ± 0.07	0.12 ± 0.08	0.15 ± 0.08	–	–	–	–	–	–
Quercetin-3-*O*-arabinoside	1.06 ± 0.13	0.98 ± 0.12	0.84 ± 0.11	0.94 ± 0.12	0.43 ± 0.07	0.61 ± 0.07	0.59 ± 0.12	0.60 ± 0.12	1.36 ± 0.11	–
Quercetin-3-*O*- galactoside	2.93 ± 0.38	2.60 ± 0.30	2.22 ± 0.28	2.40 ± 0.33	1.66 ± 0.18	2.91 ± 0.40	2.57 ± 0.27	3.14 ± 0.36	3.02 ± 0.30	2.35 ± 0.25
Quercetin-3-*O*-glucuronoside	7.27 ± 0.92	7.10 ± 0.91	6.45 ± 0.91	7.20 ± 0.86	9.17 ± 0.84	10.24 ± 0.88	9.77 ± 0.92	9.64 ± 0.69	9.31 ± 0.95	10.35 ± 0.98
Quercetin glycoside I	0.49 ± 0.13	0.48 ± 0.09	0.39 ± 0.09	0.42 ± 0.10	–	0.60 ± 0.11	–	–	0.59 ± 0.11	0.73 ± 0.12
Quercetin glycoside II	0.27 ± 0.10	0.25 ± 0.10	0.24 ± 0.09	0.29 ± 0.11	–	–	–	–	–	–
Quercetin glycoside III	0.15 ± 0.07	0.15 ± 0.07	0.14 ± 0.06	0.18 ± 0.07	–	–	–	–	–	–
Quercetin glycoside IV	0.90 ± 0.28	0.90 ± 0.27	0.86 ± 0.28	1.07 ± 0.30	–	–	–	–	–	–
Acacetin-7-*O*-glucoside	–	–	–	–	0.58 ± 0.06	0.65 ± 0.05	0.68 ± 0.09	0.62 ± 0.08	0.58 ± 0.06	0.51 ± 0.05
Kaempferide-3-*O*-glucoside	–	–	–	–	0.13 ± 0.02	–	0.08 ± 0.03	–	–	–
Flavonoid glycoside I	–	–	–	–	0.35 ± 0.03	0.30 ± 0.02	0.30 ± 0.03	0.28 ± 0.03	0.18 ± 0.02	0.23 ± 0.03
Flavonoid glycoside II	–	–	–	–	0.87 ± 0.07	0.78 ± 0.05	0.88 ± 0.08	0.83 ± 0.08	0.65 ± 0.04	0.69 ± 0.06
Flavonoid glycoside III	–	–	–	–	0.18 ± 0.02	0.23 ± 0.02	0.14 ± 0.03	–	0.08 ± 0.01	0.11 ± 0.02
Flavonoid glycoside IV	–	–	–	–	–	0.34 ± 0.05	–	–	–	–
Flavonoid glycoside V	–	–	–	–	–	0.54 ± 0.06	–	–	–	–
Flavonoid glycoside VI	–	–	–	–	–	–	0.06 ± 0.02	0.13 ± 0.01	0.12 ± 0.01	0.07 ± 0.01
Flavonoid glycoside VII	–	–	–	–	–	–	0.03 ± 0.01	–	–	0.06 ± 0.01

[a]A sum of 12 individual flavonoid aglycones (same compounds as in Salminen *et al.* 2004).

307.qxd 7/25/05 7:29 PM Page 157

of plants for herbivores (Feeny 1970; Hagermann & Robbins 1987). On the other hand, some hydrolysable tannins could act as antioxidants, thus protecting proteins and amino acids in the insects (Hagerman *et al.* 1998). The hydrolysis products of individual tannins and their metabolism within the insect digestive tract have been shown to differ, resulting negative, neglible or even positive effects for the insect (Klocke *et al.* 1986, Bernays *et al.* 1989).

3.2. Transformation of phenolic acids

Earlier we have shown that chlorogenic acid, the main phenolic acid of birch leaves, was isomerised in the alkaline gut of lepidopteran *E. autumnata* (Salminen *et al.* 2004). Similar isomerisation occurred also in guts of all sawflies tested, but there were differences in the ratios of the egested isomers between the species. For instance, while *E. autumnata* larvae excreted the three chlorogenic acid isomers (neochlorogenic acid, chlorogenic acid and the third non-foliar chlorogenic acid isomer) in approx. ratio of 1:1:1 (Salminen *et al.* 2004, unpublished data), these ratios varied in the present study from 1:1:1 (*A. amplus*) to 1:2:1 (*N. brevivalvis*) and 1:3:1 (*P. alpestris*, *P. pallipe*s, *Arge* sp., *N. viridis*). In the leaf diet the ratio of chlorogenic acid to neochlorogenic acid varied from 30:1 (first sample) to 50:1 (last sample). In addition to variable metabolism, the observed differences could suggest different pH conditions in digestive tracts of larvae, because the isomerisation of chlorogenic acid has also been observed to happen *in vitro* under basic conditions (Nagels *et al.* 1980).

Due to the potential role of chlorogenic acids in the production of *o*-quinones through auto-oxidation, or the action of the polyphenol oxidases, we also tested quantitative differences in the egestion of chlorogenic acids between studied sawfly species. Significant differences between species in relative amounts of egested chlorogenic acids ($ChiSq = 26.8$, $DF = 5$, $p < 0.0001$) were found and egestion percentages varied from 55 % in *N. brevivalvis* to 120 % in *P. pallipes*. The high egestion percentage of *P. pallipes* might be due to their specific way of consuming leaves; *P. pallipes* do not eat leaf veins and thus the concentration of compounds can be higher in faeces than in their leaf diet. Detected differences in relative amounts of egested chlorogenic acids may indicate differences in oxidation of chlorogenic acids and hence in amount of produced *o*-quinones in larvae.

In addition to chlorogenic acid isomers, gallic acid and low amount of coumaroylquinic acid were detected from faeces of all studied sawflies (Table 2). Moreover, previously undetected non-foliar phenolic acids, i.e., galloylquinic acid, protocatechuic acid and chlorogenic acid derivatives were detected. At least one of the two isomers of galloylquinic acid was detected in the faeces of all species, while protocatechuic acid was characteristic of *P. alpestris* and chlorogenic acid derivatives of *P. pallipes* only. Interestingly, gallic and quinic acids would be needed as building blocks for the non-foliar galloylquinic acids; the latter is directly available for larvae from the foliage, where free gallic acid is found only in traces. This suggests that the formation of galloylquinic acids in sawfly larvae is possible only after gallic acid is released *via* hydrolysis of galloylglucoses (see above). Protocatechuic acid in turn could originate, e.g., from the side chain reduction of caffeic acid (Waterman & Mole 1994). However, caffeic acid is not found in birch leaves, and hence would first have to be cleaved from chlorogenic acid (i.e., caffeoylquinic acid) isomers or derivatives. Alternatively, some micro-organisms are known to utilize flavonoids, and the first step in flavonoid metabolism is their conversion into different hydroxybenzoic acids, such as protocatechuic acid (Pillai & Swarup 2002).

3.3. Transformation of flavonoids

The transformations of flavonoids in sawfly larvae resembled those observed in the lepidopteran *E. autumnata* (Salminen *et al.* 2004). The main pattern was the disappearance of flavonoid aglycones and the simultaneous appearance of new flavonoid glycosides into the faeces (Table 3). Since the flavonoid aglycone levels in birch leaves decline rapidly as leaves mature (Valkama *et al.* 2004), we would have expected that the ability to detoxify flavonoid aglycones is more a property of *E. autumnata* than of sawfly larvae. Apparently the seasonal decline in foliar levels of aglycones does not significantly reduce the ability of sawflies to metabolize aglycones, since even the autumn-feeder *N. viridis* excreted detectable levels of six glycosylation products. We also tested statistical differences in egestion of non-foliar flavonoid glycosides transformed from foliar flavonoid aglycones, but there were no significant differences in relative amounts of egested glycosides ($ChiSq = 4.7$, $DF = 5$, $p < 0.4474$). The egestion percentages varied from 35 % in *A. amplus* to 44 % in *P. alpestris* with no indication of seasonal trends. In spite of similar egestion percentages, the faecal profiles of non-foliar flavonoid glycosides did vary: three of the nine compounds (acacetin-3-*O*-glucoside and flavonoid glycosides I and II) were found in the faeces of all sawflies while two compounds (flavonoid glycosides IV and V) were specific to *P. alpestris* only. Other non-foliar flavonoid glycosides showed diverse patterns, and none of them was found in the faeces of all sawfly species.

Similar to the above mentioned chlorogenic acids, the quercetin flavonoids are *o*-dihydroxylated phenolics. Thus, in principle, they also could auto-oxidize or react with polyphenol oxidases to produce harmful *o*-quinones. However, we did not find statistically significant differences between species in the relative amounts of egested quercetin glycosides ($ChiSq = 10.3$, $DF = 5$, $p < 0.0674$). Overall, the egestion percentage was rather high in all studied species (76-117 %). Similarly *E. autumnata* has reported to egest quercetin glycosides mostly unaltered (Salminen *et al.* 2004).

3.4. Possible factors behind the detected transformations

There are lots of factors which can influence on the fates of foliar phenolic compounds in larvae. As mentioned above, we know that isomerisation of chlorogenic acid is, at least partly, due to the alkaline conditions in the larvae of *E. autumnata* (Salminen *et al*. 2004). On the basis of the results of the present study, we suggest that pH-conditions in studied sawfly larvae are also alkaline. The varying ratios of isomers between the species could indicate differences in gut pH-conditions, but this preliminary conclusion needs further studies. In addition to

physiological factors, enzymes, either foliar or larval, can cause some of the detected transformations. E.g., glycosylation of flavonoids is probably due to the enzymatic activity. Glycosyl transferases should be part of birch leaf chemistry, because of number of flavonoid glycosides present in leaves (Ossipov *et al.* 1995) and after ingestion these enzymes might also act in larvae.

The role of symbiotic micro-organisms in the food metabolism of sawfly larvae is unknown to us, but their potential for creating both among- and within species variation could be substantial. Furthermore, as well as being indigenous (maternally transmitted to the offspring) the microbiota inhabiting the digestive tract may also be derived from the surrounding environment, including the host plant (Douglas & Beard 1996; Dillon & Dillon 2004). Although at least part of the observed differences between species could be explained by exogenous factors, such as the non-indigenous micro-organisms, the differences may simply reflect the evolutionary histories of the species. Pairwise coevolution (Janzen 1980; Thompson 1994) between a host plant and its herbivores can in theory produce herbivore species specific adaptations in food utilization. Furthermore, host shifts are common in many externally feeding insect herbivore taxa (e.g. Powell *et al.* 1998; Farrell & Sequeira 2004) which might increase the current variation between herbivore species sharing a host plant.

Acknowledgements

Sinikka Hanhimäki's supervision in rearing of the larvae and execution of the bioassays was invaluable. We would also like to thank Juha Järvenpää who extracted and analysed the flavonoid aglycones and our numerous field assistants for their input. This work was supported in part by grants from the Emil Aaltonen foundation (to M.L.) and from the Academy of Finland (V.O. grant no. 201073 and E.H. grants nos. 202165 and 204209).

References

Appel HM (1993) Phenolics in ecological interactions: the importance of oxidation. J Chem Ecol 19: 1521–1552

Appel HM, Martin MM (1990) Gut redox conditions in herbivorous lepidopteran larvae. J Chem Ecol 16: 3277–3290

Barbehenn RV, Martin MM (1994) Tannin sensitivity in larvae of *Malacosoma disstria* (lepidoptera): roles of peritrophic envelope and midgut oxidation. J Chem Ecol 20: 1985–2001

Barbehenn RV, Martin MM, Hagerman AE (1996) Reassessment of the roles of the peritrophic envelope and hydrolysis in protecting polyphagous grasshoppers from ingested hydrolysable tannins. J Chem Ecol 22: 1901–1919

Bernays EA, Driver GC, Bilgener M (1989) Herbivores and plant tannins. Adv Ecol Res 19: 263–302

Dillon RJ, Dillon VM (2004) The gut bacteria of insects: non pathogenic interactions. Annu Rev Entomol 49: 71–92

Douglas AE, Beard CB (1996) Microbial symbioses in the midgut of insects. Pp 419–431 *in* Lehane MJ, Billingsley PF (eds) Biology of the Insect Midgut. GB-London: Chapman & Hall

Farrell BD, Sequeira AS (2004) Evolutionary rates in the adaptive radiation of beetles on plants. Evolution 58: 1984–2001

Felton GW, Donato K, Del Vecchio RJ, Duffey SS (1989) Activation of plant foliar oxidases by insect feeding reduces nutritive quality of foliage for noctuid herbivores. J Chem Ecol 15: 2667–2694

Feeny P (1970) Seasonal changes in oak leaf tannins and nutrients as a cause of spring feeding by winter moth caterpillars. Ecology 51: 565–581

Hagerman AE, Riedl KM, Jones GA, Sovik KN, Ritchard NT, Hartzfeld PW, Riechel TL (1998) Tannins, plant phenolics, antioxidant, total antioxidant activity, oxidative damage, dietary antioxidant. J Agric Food Chem 46: 1887–1892

Hagermann AE, Robbins CT (1987) Implications of soluble tannin-protein complexes for tannin analysis and plant defence mechanisms. J Chem Ecol 13: 1243–1259

Hanhimäki S, Senn J, Haukioja E (1995) The convergence in growth of foliage-chewing insect species on individual mountain birch trees. J Anim Ecol 64: 543–552

Haukioja E (2003) Putting the insect into the birch-insect interaction. Oecologia 136: 161–168

Janzen, DH (1980) When is it coevolution? Evolution 34: 611–12

Klocke JA, van Wagenen B, Balandrin MF (1986) The ellagitannin geraniin and its hydrolysis products isolated as insect growth inhibitors from semi-arid land plants. Phytochemistry 25: 85–91

Lahtinen M, Salminen J-P, Kapari L, Lempa K, Ossipov V, Sinkkonen J, Valkama E, Haukioja E, Pihlaja K (2004) Defensive effect of surface flavonoid aglycones of *Betula pubescens* leaves against first instar *Epirrita autumnata* larvae. J Chem Ecol 30: 2257–2268

Martel J, Hanhimäki S, Kause A, Haukioja E (2001) Diversity of birch sawfly responses to seasonally atypical diets across a seasonal gradient of larval feeding periods. Entomol Exp Appl 100: 301–309

Martin JS, Martin MM (1983) Precipitation of ribulose-1,6-bis-phosphate carboxylase/oxygenase by tannic acid, quebracho, and oak foliage extracts. J Chem Ecol 9: 285–294

Nagels L, van Dongen W, de Brucker J, de Potter J (1980) High-performance liquid chromatographic separations of naturally occurring esters of phenolic acids. J Chromatogr 187: 181–187

Ossipov V, Nurmi K, Loponen J, Prokopiev N, Haukioja E, Pihlaja K (1995) HPLC isolation and identification of flavonoids from white birch *Betula pubescens* leaves. Biochem Syst Ecol 23: 213–222

Ossipov V, Nurmi K, Loponen J, Haukioja E, Pihlaja K (1996) High-performance liquid chromatographic separation and identification of phenolic compounds from leaves of *Betula pubescens* and *Betula pendula*. J Chromatogr A 721: 59–68

Pillai PVS, Swarup S (2002) Elucidation of the flavonoid catabolism pathway in Pseudomonas putida PML2 by comparative metabolic profiling. Appl Environ Microbiol 68: 143–151

Powell JA, Mitter C, Farrell B (1998) Evolution of larval food preferences in Lepidoptera. Pp 403–422 *in* Kristensen NP (ed) Handbook of zoology. Lepidoptera, Vol. 1. Systematics and evolution. DE-Berlin: De Gruyter

Riipi M, Ossipov V, Lempa K, Haukioja E, Koricheva J, Ossipova S, Pihlaja K (2002) Seasonal changes in birch leaf chemistry: are there trade-offs between leaf growth and accumulation of phenolics? Oecologia 130: 380–390

Salminen J-P, Lahtinen M, Lempa K, Kapari L, Haukioja E, Pihlaja K (2004) Metabolic modifications of birch leaf phenolics by an herbivorous insect: detoxification of flavonoid aglycones via glycosylation. Z Naturforsch 59C: 437–444

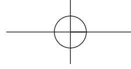
Salminen J-P, Lempa K (2002) Effects of hydrolysable tannins on an herbivorous insect: fate of individual tannins in insect digestive tract. Chemoecology 12: 203–211

Salminen J-P, Ossipov V, Haukioja E, Pihlaja K (2001) Seasonal variation in the content of hydrolysable tannins in the leaves of *Betula pubescens*. Phytochemistry 57: 15–22

Salminen J-P, Ossipov V, Loponen J, Haukioja E, Pihlaja K (1999) Characterisation of hydrolysable tannins from leaves of *Betula pubescens* by high-performance liquid chromatography – mass spectrometry. J Chrom A 864: 283–291

Salminen J-P, Ossipov V, Pihlaja K (2002) Distribution of hydrolysable tannins in the foliage of Finnish birch species. Z Naturforsch 57C: 248–256

Suomela, J, Ossipov V, Haukioja E (1995) Variation among and within mountain birch trees in foliage phenols, carbohydrates and amino acids, and in growth of *Epirrita autumnata* larvae. J Chem Ecol 21: 1421–1446



Thompson JN (1994) The Coevolutionary Process. USA-Chicago: University of Chicago Press

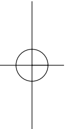
Valkama E, Salminen J-P, Koricheva J, Pihlaja K (2003) Comparative analysis of leaf trichome structure and composition of epicuticular flavonoids in Finnish birch species. Ann Bot 91: 643–655

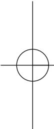
Valkama E, Salminen J-P, Koricheva J, Pihlaja K (2004) Changes in leaf trichomes and epicuticular flavonoids during leaf development in three birch taxa. Ann Bot 94: 1–10

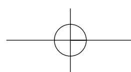
Waterman PG, Mole S (1994) Pp. 28 *in* Analysis of plant phenolic metabolites. Oxford, Blackwell scientific publications

Received 9 December 2004; accepted 7 April 2005.
Published Online First 10 June 2005.

To access this journal online:
http://www.birkhauser.ch







307.qxd 7/25/05 7:29 PM Page 160