Metabolism of poplar salicinoids by the generalist herbivore *Lymantria dispar* (Lepidoptera)

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Abstract

The survival of insect herbivores on chemically defended plants may often depend on their ability to metabolize these defense compounds. However, only little knowledge is available on how insects actually process most plant defense compounds. We investigated the metabolism of salicinoids, a major group of phenolic glycosides in poplar and willow species, by a generalist herbivore, the gypsy moth (*Lymantria dispar*). Seven salicinoid metabolites identified in gypsy moth caterpillar feces were mostly conjugates with glucose, cysteine or glycine. Two of the glucosides were phosphorylated, a feature not previously reported for insect metabolites of plant defense compounds. The origins of these metabolites were traced to specific moieties of three major poplar salicinoids ingested, salicin, salicortin and tremulacin. Based on the observed metabolite patterns we were able to deduce the initial steps of salicinoid breakdown in *L. dispar* guts, which involves cleavage of ester bonds. The conjugated molecules were effectively eliminated within 24 h after ingestion. Some of the initial breakdown products (salicin and catechol) demonstrated negative effects on insect growth and survival in bioassays on artificial diets. Gypsy moth caterpillars with *prior* feeding experience on salicinoid-containing poplar foliage converted salicinoids to the identified metabolites more efficiently than caterpillars pre-fed an artificial diet. The majority of the metabolites we identified were also produced by other common poplar-feeding insects. The conversion of plant defenses like salicinoids to a variety of water-soluble sugar, phosphate and amino acid conjugates and their subsequent excretion fits the general detoxification strategy found in insect herbivores and other animals.

Keywords: Catechol glycoside, Detoxification, Hippuric acid, *N*-acetyl cysteine conjugates, Phenolic glycosides, Phosphorylated glycosides, Poplar defense, Salicinoids.

1. Introduction

The host plants of insect herbivores often contain high quantities of chemical defense compounds. However, the mechanism by which insects avoid the toxic effects of these compounds is known in just a few cases (Després et al., 2007; Heidel-Fischer and Vogel, 2015). Insects may avoid feeding on plant parts with high concentrations of chemical defenses (Shroff et al., 2008), or rapidly excrete compounds after ingestion (Sorensen and Dearing, 2006). In addition, they may possess altered target sites that are no longer susceptible to the toxic action of the defense compound (Zhen et al., 2012), or may sequester compounds in a way that avoids intoxication (Opitz and Müller, 2009). Yet most attention has been paid to metabolic transformations that allow herbivores to detoxify defenses and excrete the resulting metabolites (Heckel, 2014; Novoselov et al., 2015). Research on herbivore detoxification of plant defense compounds and insecticides is based on the vast literature on drug metabolism in humans and other animals. Three major phases of detoxification have been described: the initial oxidation, reduction or hydrolysis of the toxin to introduce polarity or a reactive site (Phase I), conjugation with a polar molecular such as glucose, sulfate, glycine or glutathione (Phase II), and excretion (Phase III).

Insect herbivores have been frequently shown to metabolize plant defense compounds, but the identity of the metabolites is usually unknown. Genes induced upon exposure to defense compounds have been identified that encode proteins from families of known detoxification enzymes, including cytochrome P450 monooxygenases (Giraud et al., 2015), carboxylesterases (Lu et al., 2015), UDP-glucosyl transferases (Ahn et al., 2012) and glutathione S-transferases (Zhang et al., 2012), but the reaction products have rarely been identified (Heidel-Fischer and Vogel, 2015). Additionally, recent studies have raised concerns that mRNA expression profiles are not always well correlated with detoxification activities (du Rand et al., 2015). More information about the pathways by which plant defenses are metabolized would increase our knowledge about why insects are able to exploit some plants, but not others. Even if it is not always clear if a specific metabolite results from a genuine detoxification reaction, the structure can provide valuable information about the mode of action of the defense compound.

Phenolics are one of the largest group of plant defense compounds, especially in woody plants, but their metabolism by insect herbivores is little studied (Heckel, 2014). In willows and poplars, the major anti-herbivore defenses consist of the salicinoids, a group of phenolic glycosides with a core structure of a salicyl alcohol glucoside called salicin to which a variety

of organic acids are esterified (Abreu et al., 2011; Boeckler et al., 2011). The negative impact of salicinoids on the performance of generalist herbivores is well documented for various species, including *Papilio glaucus* (Lindroth, 1991), *Choristoneura conflictana* (Clausen et al., 1989), *Malacosoma disstria* and *Lymantria dispar* (Hemming and Lindroth, 1995) or *Operophtera brumata* (Ruuhola et al., 2001). Salicinoids are activated defenses, that is they are non-toxic as stored in the plant, but are activated after ingestion. Plant endogenous enzymes are often essential for the activation of plant defenses, however, in case of salicinoids activation may be carried out spontaneously (Ruuhola 2003) and by plant enzymes, such as beta glucosidases (Lindroth 1988 Hydrolysis of phenolic glycosides by beta glucosidases in *papilio glaucus* subspecies) or polyphenol oxidases (Haruta 2001 Polyphenol oxidase and herbivore defense in trembling aspen (*Populus tremuloides*): cDNA cloning, expression, and potential substrates. Salicinoids have been suggested to be activated by degradation to the toxic metabolite saligenin (reviewed in Pentzold et al., 2014) or catechol, which may oxidize to form reactive quinones (Ruuhola et al., 2001). These reactions were proposed based on the results of *in vitro* experiments (Clausen et al., 1990; Julkunen-Tiitto and Meier, 1992; Ruuhola et al., 2003) and studies with bacteria (Sonowal et al., 2013; Zhu et al., 1998) or vertebrates (Knuth et al., 2013; McLean et al., 2001), but the pathways of salicinoid metabolism in poplar-feeding arthropods are still not elucidated.

We investigated salicinoid metabolism in caterpillars of the gypsy moth (*L. dispar*), a generalist lepidopteran herbivore. Poplars are among the natural host plants of this insect and can suffer heavy defoliation during gypsy moth outbreaks (Lindroth, 1991). In this study, we identified a series of salicinoid metabolites found in the feces of gypsy moth caterpillars. By administering different types of salicinoids, the origin of the fecal metabolites was traced to specific structural features of the parent molecule. We also investigated the efficiency of salicinoid conversion to these metabolites in gypsy moth larvae, their distribution in the insect after poplar feeding, and the effect of salicinoid degradation products on caterpillar growth and survival.

2. Material and Methods

2.1 Insect, plants and artificial diet

Gypsy moth (*Lymantria dispar*) caterpillars were hatched from eggs (kindly provided by Hannah Nadel APHIS, USDA, USA) and reared on artificial gypsy moth diet (MP Biomedicals, Illkirch, France) as previously described (Boeckler et al., 2013) until they reached the 4th-5th instar. The wheat germ diet was also used for experiments where individual chemicals were tested. The diet was prepared using 1 part diet powder and 4 parts of a hot agar solution (10 g/L). When diet supplemented with chemicals was prepared, the compounds were dissolved in the agar solution before the diet powder was added. Supplemented diets were always prepared fresh immediately before the experiment and the temperature stability of additives (e.g. tremulacin) was first verified.

Populus tremula x tremuloides foliage used in our experiments originated from clonal trees of line INRA 353-38 grown in a greenhouse under conditions as previously described (Boeckler et al., 2014). For the experiments with *P. tremula x tremuloides* we always used leaves of comparably aged mature leaves.

2.2 Collection of feces for salicinoid metabolite identification

Feces extracts of gypsy moth caterpillars reared on an artificial diet devoid of salicinoids were compared to the feces extracts of individuals reared on the same diet supplemented with salicinoids (salicin, salicortin or tremulacin) or salicinoid metabolites (salicyl alcohol, catechol or benzoic acid). The compounds were either purified from *P. tremula x tremuloides* leaf material (salicortin and tremulacin) by semi-preparative HPLC as described previously (Boeckler et al., 2013) or purchased from Sigma-Aldrich (Taufkirchen, Germany, all other compounds). Each compound was added at a concentration of 38 µmol/g diet DW, which is at the lower end of the natural concentration range in poplar foliage. The diet containing each compound was apportioned into five 90 mm diameter Petri dishes (n=5) and three gypsy moth caterpillars previously starved for 24 h were added to each Petri dish. The caterpillars were allowed to feed for 5.5 h before they were transferred to a second Petri dish without diet for another 18 hours. The feces excreted within this 18 h period were then combined, flash frozen, lyophilized and then stored at -20°C until extraction and LC-MS (ion trap) analysis.

2.3 Distribution of salicinoid metabolites in gypsy moth caterpillars at different time points after ingestion and with different feeding experience

To test if the degradation of salicinoids in *Lymantria dispar* depended on previous salicinoid feeding experience, caterpillars hatched from a single egg batch were split in two groups after rearing on artificial diet to 4th-5th instar. The first group was transferred to poplar foliage (“experienced” caterpillars) while the second group remained on artificial diet (“naïve” caterpillars). The large amounts of poplar foliage required for rearing the “experienced caterpillars” in this experiment, were obtained from a mature *P. nigra* tree in Jena, Germany (50°54'38.64"N, 11°34'14.38"E) with a known salicinoid profile (Boeckler et al., 2013).

After a 4 day feeding period the caterpillars of both groups were starved for 24 h and introduced into the actual experiment. Seven “experienced” and seven “naïve” caterpillars were immediately dissected to assess initial salicinoid metabolite levels. The remaining caterpillars of both groups were placed individually in containers and offered a *P. tremula* x *tremuloides* leaf disc (Ø 24 mm) for a period of 90 min. Individuals that ate more than 30% of the leaf disc were then allowed to either digest for 90 min only or left for 24 h without provision of additional food. Twenty caterpillars of each treatment were removed for dissection after: 3 h (90 min feeding + 90 min digestion) and after 24 h (90 min feeding + 22.5 h digestion). A 90 min interval after feeding was chosen to allow initial metabolism to take place. The 24 h time point was chosen because this time was observed to be sufficient for the passage of food through 4th-5th instar gypsy moth caterpillars and we wanted to learn if all salicinoid metabolites were also eliminated within this time. For dissection, the caterpillars were kept in plastic cups on ice, anesthetized with CO₂ and then 2-3 midabdominal prolegs were removed to collect 20-100 µL hemolymph with a 200 µL pipette. Then, the head was removed and the following dissection steps were carried out in a Petri dish filled with distilled water. 1. The integument was opened by a ventral longitudinal incision to expose the digestive system. 2. The main gut was dissected and the Malpighian tubules were removed. 3. The readily distinguishable hind gut was separated from the midgut and foregut (designated together as the “main gut”). 4. The fat body and integument were discarded as they were not suitable for chemical analysis with the extraction solvent employed due to emulsion formation. 5. Each organ was thoroughly washed with distilled water, weighed and transferred to liquid nitrogen immediately. Samples were stored at -80°C until freeze-drying and dry weight recording. The extraction and analysis of salicinoid metabolites by LC-MS/MS is described below. Differences in metabolite concentrations between the two treatments and between the dissection time points were compared using a general least square (GLS) model. As a first step, we tested if the variance structure was best explained by dietary experience, dissection time or the interaction of both.

The best structure was then used in a backward model reduction approach to test the significance of dietary experience, dissection time and their interaction.

2.4 Efficiency of salicinoid conversion to identified metabolites

In this experiment the recovery of salicinoid metabolites was determined in caterpillars with and without feeding experience on salicinoid-containing diet. *L. dispar* caterpillars reared on artificial diet to 4th-5th instar were split into two groups: one group (“naïve caterpillars”) remained on artificial diet for four more days; the other group (“experienced” caterpillars) was offered poplar leaves for four days. The larvae of both groups were then starved for 48 h, a time period chosen to minimize the occurrence of residual metabolites from previous feeding bouts. Sixty caterpillars of each group were then placed individually in Petri dishes containing a moist filter paper and offered a 30 mm *P. tremula* x *tremuloides* leaf disc for 2 h. Leaf disc fresh weight was determined *prior* to placement in the Petri dishes. A subset of 20 caterpillars of both treatments that had consumed more than 20 % of their leaf disc was then selected while the other 40 individuals were discarded. The remnants of each leaf disc were immediately weighed, and then flash-frozen and freeze-dried; after reweighing, the leaves were ground to a fine powder. The selected caterpillars were allowed to excrete feces for the following 48 h, which were collected, freeze-dried and weighed. Salicinoids in the leaf discs were quantified by HPLC-UV and salicinoid metabolites in the feces were quantified by LC-MS/MS as described below. Foliar salicinoid concentrations were converted to a FW basis using the water content of the leaf disc remnants and this was used to calculate the amount of salicinoids ingested. Preliminary experiments had shown that neither salicinoid concentrations nor water content were significantly influenced by the 1.5 h feeding period (data not shown). The molar amount of excreted metabolites was calculated from their concentrations in the feces and the feces dry weight. The recovery rate of each metabolite in the feces was expressed as a percentage of the molar amount salicinoid precursor moiety ingested. Comparison between treatments was carried out after arcsine transformation using a parametric (t-test) or non-parametric test (Wilcoxon-test) depending on variance homogeneity and normal distribution.

2.5 Effect of salicinoid metabolites on caterpillar performance

Two week-old caterpillars in third instar were reared on artificial diet spiked with the salicinoid metabolites, salicin, catechol, benzoic acid or a mixture of the three, and compared to a control diet without additives. Concentrations (200 µmol/g salicin, 150 µmol/g catechol and 50 µmol/g

benzoic acid) were selected according to natural salicinoid concentrations of *Populus tremula* x *tremuloides* (Boeckler et al., 2014) assuming a quantitative release of metabolites from their respective salicinoid precursors. Ten Petri dishes of each diet were offered to groups of 7 caterpillars per dish. As we occasionally observed cannibalism during the first few days, we altered the experimental setup after three days. The number of caterpillars per dish was reduced to four and home-made plastic dividers were inserted in each of the experimental Petri dishes to ensure caterpillar separation. Every third day insects were transferred to freshly made diet with the same additives and caterpillar weight and mortality was recorded. The experiment was terminated after 15 days. Weight gain was compared using linear mixed effects models (lme). Although data were log-log transformed, the exponential growth curve of the caterpillars did not permit an analysis over the whole period of the experiment due to the inhomogeneity of variances. Therefore the comparison was restricted to data recorded between day 6 and day 15. Transformed caterpillar weights were nested within Petri dishes, and treatment and recording day (as factors) entered the model successively as fixed effects and were tested against the null model. In order to compare the treatments, they were successively combined until the minimal adequate model was achieved. Caterpillar survival was tested by a Kaplan-Meier survival analysis using the optimal error distribution (“loggaussian”).

2.6 Salicinoid conjugates in other poplar herbivore species

Eleven other poplar herbivores (Table 1) were either collected from black poplar (*P. nigra*) trees growing in a natural floodplain forest at Küstrin-Kietz, Germany (Boeckler et al., 2013) or kindly provided by hobby entomologists. All species were reared on *P. nigra* foliage and the feces of at least three individuals were collected. Feces were collected and stored at -20°C until extraction. Depending on the sample mass, feces were extracted once with 200-1000 µL aqueous MeOH (50% v:v) and qualitatively analyzed for salicinoid metabolites by LC-MS (ion-trap) and LC/MS/MS using the methods described below.

2.7 LC/MS (ion trap) analysis of salicinoids and salicinoid metabolites

Feces extracts were analyzed on an Agilent 1100 series chromatograph equipped with a reversed phase column (EC 250/4.6 Nucleodur Sphinx, RP 5 µm, Macherey-Nagel, Düren, Germany) at 25 °C and 0.2 % formic acid and acetonitrile as solvents A and B, respectively. Gradient elution with the following parameters was applied: 0-100 % B (0-50 min), 100 % B (50-55 min), 0 % B (55-60 min). After chromatographic separation, analytes were detected using an Esquire 6000 ESI-ion trap mass spectrometer (Bruker Daltonics, Bremen, Germany)

operated in alternating ionization mode and scanning a mass range from $m/z=60-1200$. Nitrogen served as nebulizer (35 psi) and drying gas (11 l/min, 330 °C). MS chromatograms of different diets were compared using the MetaboliteDetect 1.1 software provided by Bruker Daltonics (Bremen, Germany). This software subtracts a reference chromatogram (e.g. control diet) from a given chromatogram (e.g. salicin diet) and was set to calculate a differential chromatogram showing only m/z that had a two times higher intensity than in the reference chromatogram. Salicinoids and most of their metabolites were readily detected in the negative mode which was also applied for MSn experiments using the Auto-MS function of the Esquire control software.

2.8 Quantitative analysis of salicinoids by HPLC-UV

Foliar salicinoid levels were determined as described (Boeckler et al., 2013). Briefly, a 5 mg portion of leaf powder was extracted twice with 1 ml MeOH. In the first extraction step, 0.8 mg phenyl- β -glucopyranoside was added as an internal standard. Combined extracts were analyzed by HPLC as described previously and salicinoids were quantified with aid of calibration curves.

For quantitative analysis of salicinoid metabolites by LC-MS/MS, caterpillar tissues and feces were extracted whole without further treatment using 50 % aqueous MeOH containing 5 μ g/ml arbutin as internal standard. Feces and main guts were extracted twice with 750 μ L. Hemolymph, Malpighian tubules and hind guts had low samples masses (below 1 mg) and were only extracted once with 200 μ L to avoid trace analyte dilution. Each extraction step started with 5 min ultrasonication followed by 4 min of agitation on a paint shaker. Insoluble residue was spun down and the supernatant was used for chemical analysis. If necessary, the extracts were diluted 1:100 or 1:1000 with the extraction solvent to ensure that responses of abundant metabolites were within the calibration range.

Analytes were chromatographed on an Agilent 1200 series HPLC system equipped with an Agilent XDB-C-18 column (4.6 x 50 mm, 1.8 μ m). Gradient elution with 0.05 % aqueous formic acid (A) and acetonitrile (B) was applied using the following parameters: 5% B (0-0.5 min), 5-70 % B (0.5-4.5 min), 70-100 % B (4.5-6 min), 100 % B (6.0-6.5 min), 5 % B (6.5-10 min). Eluted compounds were ionized by electrospray (negative mode) and detected on an API 5000 LC/MS/MS mass spectrometer (Applied Biosystems, Carlsbad, CA, USA) using multiple reaction monitoring (MRM). MRM parameters for each analyte were optimized using standard compounds, either purchased or isolated from caterpillar feces as described below under “Isolation and identification of unknown salicinoid metabolites”. These standards were also

used to generate standard curves referenced to the internal standard arbutin. Concentrations were determined using average response factors calculated from data points within the linear range of the standard curves. In undiluted extracts, the internal standard was subject to apparent ion suppression due to its co-elution with other sugar conjugates. Thus quantification by external standard curves was carried out. MRM-parameters: (precursor ion m/z → product ion m/z ; declustering potential [V], collision energy [V]): catechol (109→91; -30, -24), catechol glucoside (270→109; -25, -24) catechol glucoside phosphate (351→79; -30, -52), salicin (285→123; -30, -18), salicin phosphate (365→79; -30, -52), hippuric acid (178→134; -20, -13), salicortin (423→123; -55, -30), tremuloidin (389→121; -20, -26), tremulacin (527→123; -55, -34), arbutin (271→161; -20, -16).

2.9 Isolation and identification of unknown salicinoid metabolites

Two metabolites, salicin phosphate and catechol glucoside phosphate, reported here for the first time, were isolated from the feces in bulk for structure elucidation. Several grams of feces containing these two metabolites were acquired by feeding 4-5th instar caterpillars with salicin (10 % DW) or catechol (1 % DW) supplemented diet. LC/MS analysis had indicated that caterpillars produced catechol glucoside phosphate (and catechol glucoside) from both salicinoids containing the HCC moiety and from catechol. Both phosphates were purified using the same protocol but were isolated separately. Approximately 1 g of feces was extracted three times with 5 ml 0.1 molar aqueous NH₃. The extract was applied to a 500 mg Chromabond HR-XA SPE column which was subsequently washed with 5 ml water and 5 ml MeOH. Residual washing solvent was removed by aspiration and compounds were eluted three times with 1 ml of a 1 M ammonia formate solution in MeOH. The eluate was fractionated on a reversed phase column (EC 250/4.6 Nucleodur Sphinx, RP 5 µm, Macherey-Nagel, Düren, Germany) using a gradient of 20 mmolar ammonium acetate and acetonitrile (ACN) at 1 ml/min. The ACN proportion was increased from 0 % to 10 % within 10 min, followed by a 3 min wash with 100 % ACN and a 3 min re-equilibration to 0 % ACN. Salicin phosphate and catechol glucoside fractions were collected from 8:48-9:12 and 9:12-9:22 min, respectively. The solvent was removed using a rotary evaporator and the solid residue was dissolved in a small portion of MeOH. This solution was again fractionated on a reversed phased column (Supelcosil LC-18-DB semi-prep, 25 cm x 10 mm x 5 µm) eluted with 10 mmol ammonium acetate (adjusted to pH 4 with acetic acid) and ACN. The initial proportion of 5 % ACN was increased to 17 % within 6 min, followed by a 2 min wash with 100 % ACN and a two min re-

equilibration with 5 % ACN. Salicin phosphate and catechol glucoside phosphate were both collected from 4:06-4:48 min. The solvent was evaporated to yield approximately 0.5 mg of the respective phosphate, which was analyzed by MS (Suppl. File 1) and NMR (Suppl. File 2).

A third unknown metabolite, the catechol N-acetylcysteine adduct was generated in vitro as part of preliminary experiments to test the reactivity of catechol. The compound was then detected in *L. dispar* feces, and its structure proven by synthesis and comparison of spectra with those of the authentic compound (Suppl. File 3).

2.10 Statistical analysis

We used the open access software package R 2.15.0 for all statistical tests. The statistical assumptions such as normal distribution and heteroscedasticity were always checked. Percent data (recovery rates) were arcsine transformed *prior* to statistical analysis.

Results

3.1 Identification of salicinoid metabolites in caterpillar feces

Altogether seven salicinoid metabolites were identified in the feces of *L. dispar* caterpillars fed salicin, salicortin and tremulacin in artificial diets at natural concentrations (**Fig. 1**). Three of these compounds, salicin phosphate (salicyl- β -D-glucoside-3-phosphate), catechol glucoside phosphate (catecholy- β -D-glucoside-3-phosphate) and the catechol N-acetylcysteine adduct, had not been previously reported. Compounds were identified based on mass spectrometry fragmentation patterns (Suppl. File 1) and NMR spectroscopy (Suppl. File 2, 3) and, for known compounds, in comparison to data of commercial standards.

The modular composition of salicinoids allowed the seven metabolites to be traced back to specific elements in their structures (Fig. 1). For example, the catechol-type metabolites were only observed when the diet contained salicortin or tremulacin, and therefore must derive from the hydroxyl cyclohexanone carboxylic acid ester (HCC) moiety that occurs only in salicortin and tremulacin, and not salicin. Hippuric acid occurred only when the caterpillars had fed on tremulacin, and thus it is most likely derived from the benzoyl ester moiety exclusive to this salicinoid. Saligenin, salicin and salicin phosphate were not specific to a single salicinoid, and consequently originate from the salicin core structure characteristic of each of these compounds.

3.2 In-vivo distribution and kinetics of salicinoid metabolism in caterpillars

To determine the kinetics of salicinoid metabolism in *L. dispar*, various tissues were analyzed before and after 90 min and 24 h, respectively, of feeding on poplar (*Populus tremula* x *tremuloides*) which contains the salicinoids salicin, salicortin and tremulacin (Boeckler et al., 2014). The metabolites identified were generally detectable in all tissues analyzed, including the main gut, hemolymph, Malpighian tubules and hindgut (**Fig. 2**). Prior to the feeding bout, none of the compounds (except the catechol N-acetylcysteine adduct) was detected in caterpillars that had not previously fed on poplar. Substantial amounts of the metabolites were detected 3 hours after feeding started, but these generally declined by 24 hours ($P < 0.001$, Fig. 2). However, catechol glucoside phosphate and salicin phosphate levels remained elevated in the hemolymph and hippuric acid remained elevated in all tissues.

The metabolism of caterpillars that had been raised on poplar was compared to caterpillars raised on an artificial diet. At the 3 h measurements, caterpillars with poplar-feeding experience contained more catechol glucoside, salicin and hippuric acid than caterpillars fed on

artificial diets, while catechol glucoside phosphate and salicin phosphate were typically higher in those fed on artificial diets (**Fig. 2**).

3.3 Efficiency of conversion of parent salicinoids to identified metabolites

The extent of breakdown of the parent salicinoids in the plant to the identified metabolites was assessed by comparing the amounts of each of the major salicinoid moieties in poplar foliage with the amounts of metabolites in gypsy moth feces. The initial foliage consisted of *Populus tremula* x *tremuloides* leaf discs containing 16.8 ± 2.1 $\mu\text{mol/g}$ salicin, 104.4 ± 3.5 $\mu\text{mol/g}$ salicortin (salicin and HCC moieties) and 42.4 ± 2.1 $\mu\text{mol/g}$ tremulacin (salicin, HCC and benzoyl moieties).

The gypsy moth feces contained no salicortin or tremulacin, but did contain salicin which may arise from the breakdown of salicortin and tremulacin to salicin or from unreacted salicin. The products of salicin metabolism, including saligenin, salicin phosphate and salicin, accounted for nearly the full amount of the salicin moiety ingested for caterpillars that had experience on poplar foliage, but accounted for only 50% of the ingested salicin moiety for caterpillars fed on artificial diet. In contrast, the recovery rate of metabolites from the HCC moiety (found in both salicortin and tremulacin) was much lower for both poplar- and artificial diet-fed caterpillars. Poplar-fed caterpillars excreted over twice as much catechol glucoside ($P < 0.001$) as artificial diet-fed caterpillars, but only half as much catechol glucoside phosphate ($P < 0.001$, **Fig. 3**).

The metabolite hippuric acid represents the benzoyl moiety of tremulacin, and accounted for 80% and 120% of the ingested benzoyl moieties in artificial diet-fed and poplar-fed caterpillars, respectively. The excess of hippuric acid in the poplar-fed caterpillars and the detection of this metabolite after ingestion of foliage from deciduous tree species without salicinoids (Suppl. Fig. 4) suggest that hippuric acid can arise not only from salicinoids like tremulacin, but also from other substances. When salicin- and HCC-derived metabolites were combined, the proportion of phosphorylated compounds was lower in the poplar-fed caterpillars (42%) as compared to the artificial diet-fed caterpillars (63%).

3.4 Effect of salicinoid metabolites on caterpillar performance

To determine the toxicity of various salicinoid degradation products and metabolites to *L. dispar*, these were added to an artificial diet, and their effect on larval performance was determined. Caterpillar weight gain on the benzoic acid and control diets was comparable and higher than weight gain on the other three treatments. On the salicin and catechol diets, weight gain was intermediate differing significantly from all the other treatments, but not from each other. Finally, caterpillars feeding on a mixed diet consisting of salicin, catechol and benzoic acid weighed significantly less than those on any other treatment (**Fig. 4**, $P < 0.001$). Caterpillar survival (excluding cannibalism) was also significantly lower on the mixed diet, while it did not differ between any of the other four treatments (**Fig. 4**, $p < 0.001$).

3.5 Salicinoid metabolites in other insect herbivores

The feces of 11 other poplar herbivores were analyzed after being fed on poplar foliage to assess if the salicinoid processing reactions found in *L. dispar* caterpillars are widespread. The feces of all tested lepidopterans but not the beetle *Chrysomela populi* contained salicin. In contrast, salicin phosphate and catechol glucoside phosphate were only present in the feces of *Orgyia antiqua*, a close relative of *L. dispar*. The catechol N-acetylcysteine adduct was excreted only by *Cerura vinula* and *Selenia dentaria*.

To determine if other simple phenolic glycosides can be phosphorylated, *L. dispar* caterpillars were fed with arbutin (hydroquinone O-glucoside) and helicin (the O-glucoside of salicylaldehyde). Both compounds were phosphorylated on their glucose residues, while administration of phenol gave both the corresponding glucoside and the phosphorylated glucoside (Suppl. Fig. 5).

4. Discussion

4.1 Salicinoid degradation in gypsy moth caterpillars involves initial ester cleavage and formation of toxic products

Salicinoids are plant anti-herbivore defenses that are thought to be activated and transformed into toxic derivatives upon plant damage or inside the herbivore (reviewed in Boeckler et al., 2011). Other types of activated defenses are known in plants, and these are typically glycosides that are converted to toxic products after sugar hydrolysis (Pentzold et al., 2014). Salicinoids do contain a β -glucoside moiety, but it is unclear if activation arises from glucose hydrolysis or from hydrolysis of ester linkages. All salicinoids except salicin contain ester bonds linking organic acids to various hydroxyl groups of the salicin core structure (**Fig. 1**).

In our investigations of salicinoid metabolism in gypsy moth caterpillars (*Lymantria dispar*), we fed foliage or artificial diet containing two compounds with such ester linkages, salicortin and tremulacin, and found no metabolites with intact ester bonds. Ester degradation is likely driven by the alkaline gut conditions found in *L. dispar* and other **lepidopterans** (Johnson and Felton, 1996; Ruuhola et al., 2001). A basic pH in the guts of herbivorous insects is believed to suppress plant glucosidases and avoid the bioactivation of glucosidic plant defenses (Pentzold et al., 2014), and may help to minimize astringency caused by tannins (reviewed by Barbehenn and Constabel, 2011; Berebaum, 1980), but favors spontaneous (non-enzymatic) degradation of ester bonds. Ester hydrolysis of salicortin yields salicin and hydroxycyclohexanone carboxylic acid (HCC), while hydrolysis of tremulacin yields salicin, HCC and benzoic acid (**Fig. 1**).

HCC is subject to further conversions in *L. dispar* as indicated by the catechol derivatives found in the feces only when salicinoids with the HCC moiety are fed upon. Previous studies provided evidence for HCC conversion to catechol after spontaneous decarboxylation and oxidation (Clausen et al., 1989). Support for alternative conversions of HCC (Clausen et al., 1990; Julkunen-Tiitto and Meier, 1992) was not obtained in the current work.

Given the formation of salicin, catechol and benzoic acid in *L. dispar* guts after ester hydrolysis, we investigated their toxicity to gypsy moth larvae in a longer-term experiment when fed at concentrations approximating those **likely to be present in the insect after ingesting** *P. tremula* x *tremuloides* foliage **assuming complete conversion of salicinoids to these metabolites**. While benzoic acid was not toxic to the gypsy moth, salicin and catechol both inhibited growth significantly. In earlier studies salicin was observed to have little if any anti-herbivore activity (Lindroth, 1988; Lindroth and Peterson, 1988), possibly because it was fed at

doses matching the low foliage concentration rather than at higher doses taking into account the salicin formed from degradation of other salicinoids, as done here.

It is currently unclear how salicin or its aglycone saligenin could act on herbivores. Saligenin could serve as an uncoupler of cell respiration, as was shown by the carboxylated analog salicylic acid (Terada, 1990). The toxicity of catechol is likely due to oxidation to quinones (Appel, 1993; Pourcel et al., 2007), which are potent electrophiles (Bittner, 2006) and can crosslink amino acids, as is the case during food browning (Friedman, 1996). In fact, the feces of *L. dispar* fed catechol containing artificial had a darker color compared to control feces, indicating that quinone-driven reactions may have occurred. In contrast to their effects on *L. dispar* growth, salicin and catechol had no effect on caterpillar survival, but a mixture of these compounds with benzoic acid caused a significant increase in mortality, indicating synergistic negative effects. In how far salicin cleavage, catechol oxidation or potential other activation reactions are driven by plant endogenous enzymes is not verifiable by our results. However, metabolite profiles in the feces of caterpillars fed poplar leaves or artificial diet devoid of plant enzymes were rather similar. Therefore the metabolic routes suggested above likely involve spontaneous reactions or are catalyzed by digestive enzymes originating from the caterpillars, but an additional role of plant enzymes is still possible.

4.2 Most identified salicinoid metabolites are products of phase II conjugation reactions

The majority of the salicinoid metabolites identified in *L. dispar* feces are formed by conjugation with glucose (catechol glucoside), phosphate (salicin phosphate, catechol glucoside phosphate) or amino acids and derivatives (hippuric acid, N-acetylcysteine catechol adduct). These are typical products of phase II detoxification reactions that increase polarity and facilitate excretion. While glucose (Ahmad and Hopkins, 1993; Ahn et al., 2011; Luque et al., 2002; Sasai et al., 2009; Wouters et al., 2014) and amino acid (Konno et al., 1996; Nijhout, 1975; Novoselov et al., 2015; Stauber et al., 2012) conjugates are well known in insects, phosphate metabolites of plant defenses are without precedent.

Given the critical metabolic roles of phosphorus in insects and literature reports that phosphorus deficiency limits insect growth (Elser et al., 2007; Visanuvmol and Bertram, 2011), it is not clear why insects would synthesize phosphate conjugates in metabolism of host plant defenses. Among the possibilities, 1) phosphorylation could serve as a metabolic tag that marks certain glycosides for efflux processes by phosphate specific transporters, an effective way of eliminating glycosides from the hemolymph against a concentration gradient. 2) Phosphorylation could avoid re-activation of glycosidic conjugates by glucosidases. Sugar

phosphorylation has a substantial impact on steric properties and polarity, and may prevent glucosidase action. 3) The excretion of excess phosphorus in the form of phosphates could be a strategy of insect herbivores to reach an optimal phosphate level in relation to other nutrients. For example, tight maintenance of phosphate homeostasis has been observed in the grasshopper *Oedaleus asiaticus* (Zhang et al., 2014). The amount of phosphorus used in producing salicinoid metabolites makes up a substantial portion of the overall phosphorus budget of *L. dispar*. *P. nigra* foliage contains an average of 100 $\mu\text{mol/g}$ phosphorus-, while salicin phosphate and catechol glucoside phosphate in the feces of *L. dispar* caterpillars fed *P. tremula* x *tremuloides* add up to approximately 50 $\mu\text{mol/g}$ (Boeckler, unpublished results). Assuming that poplar foliage has an approximate digestibility of 0.5 based on dry weight (Kinney et al., 1997), our results suggest that under natural conditions gypsy moth larvae expend up to one quarter of their phosphorus uptake on salicinoid detoxification. While the amount of phosphorus in the artificial diet may have been higher than that in *P. tremula* x *tremuloides* foliage, the overall amounts of phosphorylated metabolites formed in *L. dispar* larvae feeding on artificial diet were not greater than those formed in foliage-fed larvae. Only the relative abundance of the two phosphorylated products was altered. *L. dispar* was able to phosphorylate other phenolic glycosides and phenol offered in artificial diet (Suppl. File 5), suggesting that the ability to produce phosphate conjugates of phenolic compounds may be a general response in this and perhaps other insect species.

Among the amino acid conjugates found in this study, the glycine conjugate of benzoic acid (hippuric acid) reported here has long been known from other insects (Nijhout, 1975; Shyamala, 1964), and it has been speculated that some Lepidopteran species maintain high concentrations of glycine in their guts to detoxify dietary aromatic acids (Konno et al., 1996). The N-acetylcysteine conjugate of catechol has not been previously reported in insects, and probably arises via initial conjugation with glutathione followed by peptidase cleavage of the γ -glutamate and glycine residues of glutathione and subsequent N-acetylation, along the so-called mercapturic acid pathway, as described in other insects (Gloss et al., 2014) and mammals (Cristofori et al., 2015). The conjugation of glutathione with another group of plant defense compounds, the isothiocyanates, is well known (Gloss et al., 2014; Schramm et al., 2012).

Our work on gypsy moth metabolism of salicinoids is one of the first to report on phenolic processing in a generalist insect herbivore. To determine if the seven salicinoid metabolites formed by gypsy moth are also produced in other insect herbivores, we analyzed the feces of 11 other insect herbivores, 10 lepidopterans and one coleopteran, after feeding on

poplar. All of the metabolites identified here, except for the phosphates, were detected in all species (**Table 1**), but the phosphates were only present in the feces of the rusty tussock moth (*Orgyia antiqua*), a close relative of *Lymantria dispar*.

4.3 Localization and transport of salicinoid metabolites in gypsy moth

Our results on the distribution of salicinoid metabolites in various organs of gypsy moth larvae shed light on the localization of the metabolite-forming reactions and subsequent transport processes. After initial ester cleavage of salicinoids in the gut lumen, the phase II conjugation reactions must take place within cells because these reactions require intracellular enzymes and high energy cellular intermediates, such as UDPG and glutathione, and would not be favored under the typical pH environment of the insect gut. The probable location is the epithelial cells lining the gut lumen, site of many metabolic reactions associated with nutrient digestion, assimilation and detoxification (Appel and Martin, 1990; Novoselov et al., 2015). After conjugation, the polar products can be transported back into the gut lumen for excretion or loaded in the hemolymph. The release of conjugates in the gut lumen reduces their proximity to internal metabolism, but hemolymph loading may prevent reaction by digestive enzymes. Both processes appear to occur in *L. dispar* since both gut tissue and hemolymph contained substantial amounts of salicinoid metabolites (**Fig. 2**). In insects, the elimination of waste products from the hemolymph is carried out by the Malpighian tubules (Chahine and O'Donnell, 2011), but so far there is little direct evidence for the function of these organs in plant defense detoxification. In the gypsy moth, most salicinoid conjugates were concentrated at higher levels in the Malpighian tubules as compared to the hemolymph (**Fig. 2**), indicating that these organs have a major responsibility for translocation to the hindgut, in which even higher concentrations of conjugates were observed. Deposition in the hindgut was followed finally by elimination in the feces. Whatever the route to the hindgut, *L. dispar* caterpillars metabolized salicinoids and eliminated most of the identified metabolites in less than 24 hours. Alternatively, salicinoid metabolism may be carried out within the cells of endosymbiotic microbes, which could play an important role in detoxification of plant defenses in insects (Engel and Moran, 2013). In *L. dispar*, *Acinetobacter* species derived from the host plant have been demonstrated to metabolize salicinoids (Mason et al., 2014; Mason et al., 2015). Insect metabolism of plant defenses is not a static process, but is conditioned by initial exposure to a compound such that metabolism is elevated upon subsequent contact (Dugravot et al., 2004; Falk and Gershenzon, 2007; Li et al., 2004; Zhang et al., 2012). Such flexibility is considered an adaptive mechanism for reducing metabolic costs in generalist herbivores, such

as gypsy moth caterpillars, that often switch host plants during larval development. In this study, most salicinoid metabolites were formed at higher levels in larvae that had prior experience with poplar foliage compared to larvae that had fed only on artificial diet (**Fig. 3**). Elimination rates were also affected by feeding experience. Of the compounds remaining at high levels in the hemolymph after 3 h, salicin phosphate and catechol glucoside phosphate were three times less abundant in larvae that had fed on poplar suggesting a much higher elimination rate in experienced insects. Poplar-experienced caterpillars also eliminated salicin more efficiently than caterpillars fed on artificial diet, although the recovery of the HCC moiety was similar. For both the salicin and HCC moieties, recovery was sometimes low, less than 50% and 30%, respectively, depending on treatment. Hence there must be other metabolic sinks in *L. dispar* caterpillars than the observed conjugates. Rats have been reported to oxidize salicin (Akao et al., 2002) and specialized leaf beetles convert this compound to salicylaldehyde (Strauss et al., 2013), which they use to deter predators. For HCC, two metabolic routes have been suggested in the literature, which both involve irreversible covalent binding to proteins. Clausen et al (1990) found that HCC can form quinone methides which attack and deactivate glucosidases (Zhu et al., 1998). Alternatively, if catechol is derived from the HCC moiety, as shown in our work and previously (Pearl and Darling, 1970; Ruuhola et al., 2003), this compound could also form quinones and react with proteins (Appel, 1993).

Further work is needed to document the metabolism of salicinoids in gypsy moth more completely. The use of isotopically-labeled precursors might facilitate the identification of the remaining major metabolites, as well as make progress in deducing the mode of action of salicinoids in herbivores like the gypsy moth. Little is known about the molecular targets of salicinoids and other plant phenolic defense compounds in insect herbivores. Once the principal pathways of salicinoid processing are elucidated, researchers can turn to identifying the genes and enzymes involved. Characterization of the properties of these genes and enzymes and their evolutionary origin should give many new insights into plant-herbivore interactions occurring in a whole range of poplars, willows and other plants that produce phenolics.

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References

- Abreu, I.N., Ahnlund, M., Moritz, T., Albrechtsen, B.R., 2011. UHPLC-ESI/TOFMS determination of salicylate-like phenolic glycosides in *Populus tremula* leaves. J. Chem. Ecol. 37, 857-870.
- Ahmad, S.A., Hopkins, T.L., 1993. β -glucosylation of plant phenolics by phenol β -glucosyltransferase in larval tissues of the tobacco hornworm, *Manduca sexta* (L.). Insect Biochem. Mol. Biol. 23, 581-589.
- Ahn, S.J., Badenes-Perez, F.R., Reichelt, M., Svatos, A., Schneider, B., Gershenzon, J., Heckel, D.G., 2011. Metabolic detoxification of capsaicin by UDP-glycosyltransferase in three *Helicoverpa* species. Arch. Insect Biochem. Physiol. 78, 104-118.
- Ahn, S.J., Vogel, H., Heckel, D.G., 2012. Comparative analysis of the UDP-glycosyltransferase multigene family in insects. Insect Biochem. Mol. Biol. 42, 133-147.
- Akao, T., Yoshino, T., Kobashi, K., Hattori, M., 2002. Evaluation of salicin as an antipyretic prodrug that does not cause gastric injury. Planta Med. 68, 714-718.
- Appel, H.M., 1993. Phenolics in ecological interactions: the importance of oxidation. J. Chem. Ecol. 19, 1521-1552.
- Appel, H.M., Martin, M.M., 1990. Gut redox conditions in herbivorous lepidopteran larvae. J. Chem. Ecol. 16, 3277-3290.
- Barbehenn, R.V., Constabel, C.P., 2011. Tannins in plant-herbivore interactions. Phytochemistry 72, 1551-1565.
- Berenbaum, M., 1980. Adaptive significance of midgut PH in larval lepidoptera. Amer. Nat. 115, 138-146.
- Bittner, S., 2006. When quinones meet amino acids: chemical, physical and biological consequences. Amino Acids 30, 205-224.
- Boeckler, G.A., Gershenzon, J., Unsicker, S.B., 2011. Phenolic glycosides of the Salicaceae and their role as anti-herbivore defenses. Phytochemistry 72, 1497-1509.
- Boeckler, G.A., Gershenzon, J., Unsicker, S.B., 2013. Gypsy moth caterpillar feeding has only a marginal impact on phenolic compounds in old-growth black poplar. J. Chem. Ecol. 39, 1301-1312.
- Boeckler, G.A., Towns, M., Unsicker, S.B., Mellway, R.D., Yip, L., Hilke, I., Gershenzon, J., Constabel, C.P., 2014. Transgenic upregulation of the condensed tannin pathway in poplar leads to a dramatic shift in leaf palatability for two tree-feeding Lepidoptera. J. Chem. Ecol. 40, 150-158.

560 Chahine, S., O'Donnell, M.J., 2011. Interactions between detoxification mechanisms and
 561 excretion in Malpighian tubules of *Drosophila melanogaster*. J. Exp. Biol. 214, 462-468.
 562 Clausen, T.P., Keller, J.W., Reichardt, P.B., 1990. Aglycone fragmentation accompanies β -
 563 glucosidase catalyzed hydrolysis of salicortin, a naturally-occurring phenol glycoside.
 564 Tetrahedron Lett. 31, 4537-4538.
 565 Clausen, T.P., Reichardt, P.B., Bryant, J.P., Werner, R.A., Post, K., Frisby, K., 1989. Chemical
 566 model for short-term induction in quaking aspen (*Populus tremuloides*) foliage against
 567 herbivores. J. Chem. Ecol. 15, 2335-2346.
 568 Cristofori, P., Sauer, A.V., Trevisan, A., 2015. Three common pathways of nephrotoxicity
 569 induced by halogenated alkenes. Cell Biol. Toxicol. 31, 1-13.
 570 Després, L., David, J.P., Gallet, C., 2007. The evolutionary ecology of insect resistance to plant
 571 chemicals. Trends Ecol. Evol. 22, 298-307.
 572 du Rand, E.E., Smit, S., Beukes, M., Apostolides, Z., Pirk, C.W.W., Nicolson, S.W., 2015.
 573 Detoxification mechanisms of honey bees (*Apis mellifera*) resulting in tolerance of dietary
 574 nicotine. Sci. Rep. 5: 11779.
 575 Dugravot, S., Thibout, E., Abo-Ghaila, A., Huignard, J., 2004. How a specialist and a non-
 576 specialist insect cope with dimethyl disulfide produced by *Allium porrum*. Entomol. Exp. Appl.
 577 113, 173-179.
 578 Elser, J.J., Bracken, M.E.S., Cleland, E.E., Gruner, D.S., Harpole, W.S., Hillebrand, H., Ngai,
 579 J.T., Seabloom, E.W., Shurin, J.B., Smith, J.E., 2007. Global analysis of nitrogen and
 580 phosphorus limitation of primary producers in freshwater, marine and terrestrial ecosystems.
 581 Ecol. Lett. 10, 1135-1142.
 582 Engel, P., Moran, N.A., 2013. The gut microbiota of insects - diversity in structure and
 583 function. FEMS Microbiol. Rev. 37, 699-735.
 584 Falk, K.L., Gershenzon, J., 2007. The desert locust, *Schistocerca gregaria*, detoxifies the
 585 glucosinolates of *Schouwia purpurea* by desulfation. J. Chem. Ecol. 33, 1542-1555.
 586 Friedman, M., 1996. Food browning and its prevention: An overview. J. Agric. Food Chem.
 587 44, 631-653.
 588 Giraudo, M., Hilliou, F., Fricaux, T., Audant, P., Feyereisen, R., Le Goff, G., 2015.
 589 Cytochrome P450s from the fall armyworm (*Spodoptera frugiperda*): responses to plant
 590 allelochemicals and pesticides. Insect Mol. Biol. 24, 115-128.
 591 Gloss, A.D., Vassao, D.G., Hailey, A.L., Nelson Dittrich, A.C., Schramm, K., Reichelt, M.,
 592 Rast, T.J., Weichsel, A., Cravens, M.G., Gershenzon, J., Montfort, W.R., Whiteman, N.K.,

2014. Evolution in an ancient detoxification pathway is coupled with a transition to herbivory in the Drosophilidae. *Mol. Biol. Evol.* 31, 2441-2456.

Heckel, D.G., 2014. Insect detoxification and sequestration strategies. *Annu. Plant Rev.* 47, 77-114.

Heidel-Fischer, H.M., Vogel, H., 2015. Molecular mechanisms of insect adaptation to plant secondary compounds. *Curr. Opin. Insect Sci.* 8, 8-14.

Hemming, J.D.C., Lindroth, R.L., 1995. Intraspecific variation in aspen phytochemistry: Effects on performance of gypsy moth and forest tent caterpillars. *Oecologia* 103, 79-88.

Johnson, K.S., Felton, G.W., 1996. Potential influence of midgut pH and redox potential on protein utilization in insect herbivores. *Arch. Insect Biochem. Physiol.* 32, 85-105.

Julkunen-Tiitto, R., Meier, B., 1992. The enzymatic decomposition of salicin and its derivatives obtained from Salicaceae species. *J. Nat. Prod.* 55, 1204-1212.

Kinney, K.K., Lindroth, R.L., Jung, S.M., Nordheim, E.V., 1997. Effects of CO₂ and NO₃-availability on deciduous trees: Phytochemistry and insect performance. *Ecology* 78, 215-230.

Knuth, S., Abdelsalam, R.M., Khayyal, M.T., Schweda, F., Heilmann, J., Kees, M.G., Mair, G., Kees, F., Jurgensliemk, G., 2013. Catechol conjugates are *in vivo* metabolites of *Salicis cortex*. *Planta Med.* 79, 1489-1494.

Konno, K., Hirayama, C., Shinbo, H., 1996. Unusually high concentration of free glycine in the midgut content of the silkworm, *Bombyx mori*, and other lepidopteran larvae. *Comp. Biochem. Physiol. A* 115, 229-235.

Li, W., Zangerl, A.R., Schuler, M.A., Berenbaum, M.R., 2004. Characterization and evolution of furanocoumarin-inducible cytochrome P450s in the parsnip webworm, *Depressaria pastinacella*. *Insect Mol. Biol.* 13, 603-613.

Lindroth, R.L., 1988. Effects of quaking aspen compounds catechol, salicin and isoniazid on 2 subspecies of tiger swallowtails. *Am. Midl. Nat.* 119, 1-6.

Lindroth, R.L., 1991. Biochemical ecology of aspen-Lepidoptera interactions. *J. Kansas Entomol. Soc.* 64, 372-380.

Lindroth, R.L., Peterson, S.S., 1988. Effects of plant phenols on performance of southern armyworm larvae. *Oecologia* 75, 185-189.

Lu, F.G., Fu, K.Y., Li, Q., Guo, W.C., Ahmat, T., Li, G.Q., 2015. Identification of carboxylesterase genes and their expression profiles in the Colorado potato beetle *Leptinotarsa decemlineata* treated with fipronil and cyhalothrin. *Pest. Biochem. Physiol.* 122, 86-95.

Luque, T., Okano, K., O'Reilly, D.R., 2002. Characterization of a novel silkworm (*Bombyx mori*) phenol UDP-glucosyltransferase. *Eur. J. Biochem.* 269, 819-825.

627 Mason, C.J., Couture, J.J., Raffa, K.F., 2014. Plant-associated bacteria degrade defense
628 chemicals and reduce their adverse effects on an insect defoliator. *Oecologia* 175, 901-910.

629 Mason, C.J., Rubert-Nason, K.F., Lindroth, R.L., Raffa, K.F., 2015. Aspen defense chemicals
630 influence midgut bacterial community composition of gypsy moth. *J. Chem. Ecol.* 41, 75-84.

631 McLean, S., Pass, G.J., Foley, W.J., Brandon, S., Davies, N.W., 2001. Does excretion of
632 secondary metabolites always involve a measurable metabolic cost? Fate of plant antifeedant
633 salicin in common brushtail possum, *Trichosurus vulpecula*. *J. Chem. Ecol.* 27, 1077-1089.

634 Nijhout, H.F., 1975. Excretory role of the midgut in larvae of the tobacco hornworm, *Manduca*
635 *sexta* (L.). *J. Exp. Biol.* 62, 221-230.

636 Novoselov, A., Becker, T., Pauls, G., von ReuSs, S.H., Boland, W., 2015. *Spodoptera littoralis*
637 detoxifies neurotoxic 3-nitropropanoic acid by conjugation with amino acids. *Insect Biochem,*
638 *Mol. Biol.* 63, 97-103.

639 Opitz, S.E.W., Müller, C., 2009. Plant chemistry and insect sequestration. *Chemoecology* 19,
640 117-154.

641 Pearl, I.A., Darling, S.F., 1970. The structures of salicortin and tremulacin. *Tetrahedron Lett.*
642 11, 3827-3830.

643 Pentzold, S., Zagrobelny, M., Rook, F., Bak, S., 2014. How insects overcome two-component
644 plant chemical defence: plant β -glucosidases as the main target for herbivore adaptation. *Biol.*
645 *Rev.* 89, 531-551.

646 Pourcel, L., Routaboul, J.M., Cheynier, V., Lepiniec, L., Debeaujon, I., 2007. Flavonoid
647 oxidation in plants: from biochemical properties to physiological functions. *Trends Plant Sci.*
648 12, 29-36.

649 Ruuhola, T., Julkunen-Tiitto, R., Vainiotalo, P., 2003. In vitro degradation of willow
650 salicylates. *J. Chem. Ecol.* 29, 1083-1097.

651 Ruuhola, T., Tikkanen, O.P., Tahvanainen, J., 2001. Differences in host use efficiency of
652 larvae of a generalist moth, *Operophtera brumata* on three chemically divergent *Salix* species.
653 *J. Chem. Ecol.* 27, 1595-1615.

654 Sasai, H., Ishida, M., Murakami, K., Tadokoro, N., Ishihara, A., Nishida, R., Mori, N., 2009.
655 Species-specific glucosylation of DIMBOA in larvae of the rice armyworm. *Biosci. Biotech.*
656 *Biochem.* 73, 1333-1338.

657 Schramm, K., Vassao, D.G., Reichelt, M., Gershenzon, J., Wittstock, U., 2012. Metabolism of
658 glucosinolate-derived isothiocyanates to glutathione conjugates in generalist lepidopteran
659 herbivores. *Insect Biochem. Mol. Biol.* 42, 174-182.

660 Shroff, R., Vergara, F., Muck, A., Svatos, A., Gershenzon, J., 2008. Nonuniform distribution of
 661 glucosinolates in *Arabidopsis thaliana* leaves has important consequences for plant defense.
 662 PNAS 105, 6196-6201.

663 Shyamala, M.B., 1964. Detoxification of benzoate by glycine conjugation in the silkworm,
 664 *Bombyx mori* L. J. Insect Physiol. 10, 385-391.

665 Sonowal, R., Nandimath, K., Kulkarni, S.S., Koushika, S.P., Nanjundiah, V., Mahadevan, S.,
 666 2013. Hydrolysis of aromatic beta-glucosides by non-pathogenic bacteria confers a chemical
 667 weapon against predators. Proc. R. Soc. B-Biol. Sci. 280, 20130721.

668 Sorensen, J.S., Dearing, M.D., 2006. Efflux transporters as a novel herbivore
 669 countermechanism to plant chemical defenses. J. Chem. Ecol. 32, 1181-1196.

670 Stauber, E.J., Kuczka, P., van Ohlen, M., Vogt, B., Janowitz, T., Piotrowski, M., Beuerle, T.,
 671 Wittstock, U., 2012. Turning the 'mustard oil bomb' into a 'cyanide bomb': aromatic
 672 glucosinolate metabolism in a specialist insect herbivore. PLoS One 7, e35545.

673 Strauss, A.S., Peters, S., Boland, W., Burse, A., 2013. ABC transporter functions as a
 674 pacemaker for sequestration of plant glucosides in leaf beetles. Elife 2, e01096.

675 Terada, H., 1990. Uncouplers of oxidative phosphorylation. Environ. Health Perspect. 87, 213-
 676 218.

677 Visanuvimol, L., Bertram, S.M., 2011. How dietary phosphorus availability during
 678 development influences condition and life history traits of the cricket, *Acheta domesticus*. J.
 679 Insect Sci. 11: 63.

680 Wouters, F.C., Reichelt, M., Glauser, G., Bauer, E., Erb, M., Gershenzon, J., Vassao, D.G.,
 681 2014. Reglucosylation of the benzoxazinoid DIMBOA with inversion of stereochemical
 682 configuration is a detoxification strategy in lepidopteran herbivores. Angew. Chem. 126,
 683 11502–11506.

684 Zhang, Y.E., Ma, H.J., Feng, D.D., Lai, X.F., Chen, Z.M., Xu, M.Y., Yu, Q.Y., Zhang, Z.,
 685 2012. Induction of detoxification enzymes by quercetin in the silkworm. J. Econ. Entomol.
 686 105, 1034-1042.

687 Zhang, Z.J., Elser, J.J., Cease, A.J., Zhang, X.M., Yu, Q., Han, X.G., Zhang, G.M., 2014.
 688 Grasshoppers regulate N:P stoichiometric homeostasis by changing phosphorus contents in
 689 their frass. PLoS One 9, e103697.

690 Zhen, Y., Aardema, M.L., Medina, E.M., Schumer, M., Andolfatto, P., 2012. Parallel
 691 molecular evolution in an herbivore community. Science 337, 1634-1637.

692 Zhu, J.J., Withers, S.G., Reichardt, P.B., Treadwell, E., Clausen, T.P., 1998. Salicortin: a
693 repeat-attack new-mechanism-based *Agrobacterium faecalis* beta-glucosidase inhibitor.
694 Biochem. J. 332, 367-371.

695 **Tables**

696 **Table 1:** Occurrence of *Lymantria dispar* salicinoid metabolites in other insect herbivores species that feed on poplar. All the lepidopterans studied
697 were analyzed in their larval stages, while *C. populi* was analyzed as an adult. Feces extracts were analyzed by LC/MS and LC/MS/MS. + detected, -
698 not detected, ACC, N-acetylcysteine.

Family	Species	Saligenin	Salicin	Salicin phosphate	Catechol glucoside	Catechol glucoside phosphate	Catechol ACC adduct	Hippuric acid
Lymantriidae	<i>Lymantria dispar</i>	+	+	+	+	+	+	+
Geometridae	<i>Selenia dentaria</i>	+	+	-	+	-	+	+
Lasiocampidae	<i>Macrothylacia rubi</i>	+	+	-	+	-	-	+
Lymantriidae	<i>Orgyia antiqua</i>	+	+	+	+	+	-	+
Noctuidae	<i>Scoliopteryx libatrix</i>	+	+	-	+	-	-	+
Noctuidae	<i>Catocala nupta</i>	+	+	-	+	-	-	+
Noctuidae	<i>Conistra vacinii</i>	+	+	-	+	-	-	+
Notodontidae	<i>Phalera bucephala</i>	+	+	-	+	-	-	+
Notodontidae	<i>Notodonta ziczac</i>	+	+	-	+	-	-	+
Notodontidae	<i>Cerura vinula</i>	+	+	-	+	-	+	+
Sphingidae	<i>Laothoe populi</i>	+	+	-	+	-	-	+
Chrysomelidae (Coleoptera)	<i>Chrysomela populi</i>	+	-	-	+	-	-	+

699

Figure legends

Figure 1

Representative LC/MS/MS chromatograms of feces extracts of *Lymantria dispar* caterpillars fed artificial diet (control) or artificial diet spiked with salicin, salicortin or tremulacin. Each chromatogram contains the superimposed multiple reaction monitoring (MRM) ion traces of the seven salicinoid metabolites identified. The scaling is uniform within the traces for each metabolite but not between metabolites because of large differences in metabolite abundance. Structures of the metabolites and their corresponding precursors and metabolic intermediates are shown next to the chromatograms. Numbers indicate the peak identity (1 = salicin phosphate, 2 = salicin, 3 = saligenin, 4 = catechol glucoside phosphate, 5 = catechol glucoside, 6 = catechol acetyl cysteine adduct, 7 = hippuric acid). HCC, hydroxyl cyclohexanone carboxylic acid.

Figure 2 Salicinoid metabolite concentrations in different organs of gypsy moth caterpillars before (0 h), shortly after (3 h) and long after (24 h) the beginning of a 90 min feeding period on *Populus tremula* x *tremuloides* foliage. Naïve caterpillars (white bars) had no preliminary experience with dietary salicinoids while experienced (grey bars) caterpillars had been allowed to eat poplar foliage for 4 days. Bars represent mean $\pm$ SE of 6 (0 h) or 20 replicates (3 h and 24 h), respectively. Asterisks indicate significance of general least square model testing the effect of diet treatment (D), time after feeding (T) and their interaction (D x T), * $P \geq 0.05$, ** $P \geq 0.01$, ***- $P \geq 0.001$, ns = non significant.

Figure 3 Recovery of salicinoid metabolites from *Lymantria dispar* caterpillars after a 2 h feeding bout on *P. tremula* x *tremuloides* foliage. Metabolites are grouped according to the structural moiety they are derived from (Fig. 1). Caterpillars had been previously fed for 4 days on poplar foliage (gray bars) or artificial diet (white bars). Recoveries are calculated as percent of the total amount of each moiety present in foliage ingested on a molar basis. The bars labeled “unrecovered” indicate the percentage of each moiety not recovered in the total pool of identified metabolites. Bars represent the mean of 20 replicates $\pm$ SE. Asterisks indicate significant differences between the treatments, based on a student’s T-test (* $p \geq 0.05$, ** $p \geq 0.01$, ***- $p \geq 0.001$, ns = non significant). ACC-catechol, N-acetylcysteine catechol conjugate.

Figure 4 *Lymantria dispar* caterpillar weight gain and survival on artificial diet supplement with salicin (200 $\mu\text{mol/g}$ FW), catechol (150 $\mu\text{mol/g}$ FW), benzoic acid (50 $\mu\text{mol/g}$ FW), a mixture of the three compounds and a control treatment without additives. Weight gain was compared using a linear mixed effect model and was significantly influenced by time and diet (linear mixed effect model, both $P < 0.001$). Significant differences among individual treatments determined by model reduction are indicated as superscript letters in the legend. Weight gain graphs represent the mean $\pm$ SE of 10 replicate Petri dishes. Survival was analyzed by Kaplan-Meier analysis and the results are displayed as superscript letters.

Tables

Table 1: Occurrence of *Lymantria dispar* salicinoid metabolites in other insect herbivores species that feed on poplar. All are lepidopteran species, except *Chrysomela populi*, which is a coleopteran. All the lepidopterans studied were analyzed in their larval stages, while *C. populi* was analyzed as an adult. Feces extracts were analyzed by LC/MS and LC/MS/MS. + detected, - not detected, ACC, N-acetylcysteine.

Family	Species	Saligenin	Salicin	Salicin phosphate	Catechol glucoside	Catechol glucoside phosphate	Catechol ACC adduct	Hippuric acid
Lymantriidae	<i>Lymantria dispar</i>	+	+	+	+	+	+	+
Geometridae	<i>Selenia dentaria</i>	+	+	-	+	-	+	+
Lasiocampidae	<i>Macrothylacia rubi</i>	+	+	-	+	-	-	+
Lymantriidae	<i>Orgyia antiqua</i>	+	+	+	+	+	-	+
Noctuidae	<i>Scoliopteryx libatrix</i>	+	+	-	+	-	-	+
Noctuidae	<i>Catocala nupta</i>	+	+	-	+	-	-	+
Noctuidae	<i>Conistra vacinii</i>	+	+	-	+	-	-	+
Notodontidae	<i>Phalera bucephala</i>	+	+	-	+	-	-	+
Notodontidae	<i>Notodonta ziczac</i>	+	+	-	+	-	-	+
Notodontidae	<i>Cerura vinula</i>	+	+	-	+	-	+	+
Sphingidae	<i>Laothoe populi</i>	+	+	-	+	-	-	+
Chrysomelidae (Coleoptera)	<i>Chrysomela populi</i>	+	-	-	+	-	-	+

Figure 1

FIGURES

Figure 2

Figure 3

Figure 4