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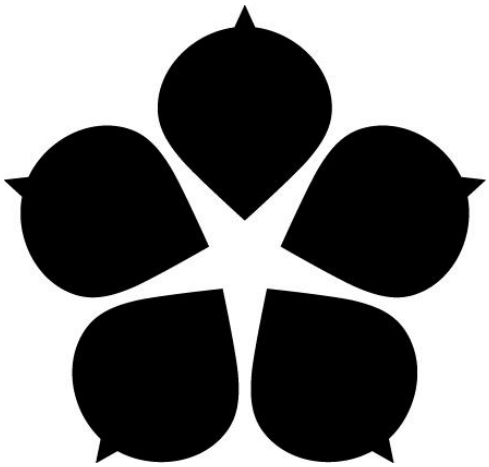

Insect herbivores drive the loss of unique chemical defense in willows

RNDr. Thesis

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Annotation
The thesis examines the effects of chemical and mechanical defensive traits on insects in a local community of 11 Salicaceae species growing in sympatry. The results repeated loss of willow specialized chemical defense. This could be due to its low protective value and high energy costs. Our study thus shows that the balance between costs and benefits of defensive traits is not necessarily in favor of specialized defenses and illustrates a process, which may lead to the reduction in a defensive trait.

Declaration [in Czech]
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V Českých Budějovicích, 20. 10. 2015

Martin Volf

Author contribution

The author of the thesis sampled the insect data, measured the host-plant traits and chemistry, obtained the host-plant DNA sequences using molecular methods, reconstructed the host-plant phylogeny, designed the hypotheses, analyzed the data, and wrote the first draft of the manuscript.

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Insect herbivores drive the loss of unique chemical defense in willows

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Abstract

Throughout the course of their evolution, plants have acquired a wide range of chemical and mechanical defenses to protect against herbivores. Ehrlich & Raven's coevolutionary theory suggests that this diversification of defensive traits is driven by the strong impact of novel traits on insect herbivores. However, the impact of plant defenses on insects is difficult to compare between related plant species due to variation in environmental and biotic conditions. We standardized these factors as far as possible by analyzing the effects of chemical and mechanical defensive traits on insects in a local community of 11 Salicaceae species growing in sympatry, and their leaf-chewing herbivores. Defensive traits (salicylates, flavonoids, tannins, trichomes, and leaf toughness) were generally not inter-correlated, with the exception of a negative correlation between salicylates and trichomes. The content of salicylates, a novel group of defensive metabolites in the Salicaceae, was correlated with low herbivore diversity and high host specificity. Despite these effects, the phylogeny of the studied species shows loss of salicylates in some *Salix* species instead of their further diversification. This could be due to salicylates not decreasing the overall abundance of herbivores, despite accounting for up to 22% of the dry leaf mass and therefore being costly. The defense of low-salicylate willow species is thus probably maintained by other defensive traits, such as trichomes. Our study shows that the balance between costs and benefits of defensive traits is not necessarily in favor of novel compounds and illustrates a process, which may lead to the reduction in a defensive trait.

Introduction

In their coevolutionary theory, Ehrlich & Raven (1964) proposed that an arms race between plants and herbivorous insects leads to the continued diversification of defensive traits, driven by the strong impact of novel traits on herbivores. The insects act as a selective pressure promoting increased plant defense (Benderoth et al., 2006), and many novel defensive traits appear during the course of plant evolution (Fucile et al., 2008; Kliebenstein & 2012). Although the evolution of plant defenses was studied in several systems (e.g., Agrawal & Fishbein, 2008; Becerra et al., 2009; Kursar et al., 2009; Agrawal et al., 2012), explaining the evolution of plant secondary metabolites in the coevolutionary process requires further attention as different groups of secondary metabolites exhibit different evolutionary patterns. For example, diversification of secondary metabolites has been found in the genus *Bursera* (Becerra et al., 2009). On the other hand, the support for theoretical predictions of ever-expanding and diversifying defenses in *Asclepias* spp. is more equivocal, as the presence of cardenolides appears to have decreased with phylogenetic diversification (Agrawal & Fishbein, 2008).

The reduction or loss of secondary metabolites is expected, if they become ineffective in anti-herbivore defense or too costly. The benefits of defensive traits are defined by a combination of their anti-herbivore efficacy and the impact of herbivores on unprotected plants. Previous studies analyzed this cost–benefit balance by focusing on the overall abundance of herbivores and/or plant damage caused by them, while paying little attention to the herbivore species causing it (Coley et al., 2005; Agrawal &

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Fishbein, 2008). We suggest that it is important to analyze herbivore community composition and life history traits, as individual defensive traits may have different effects on specialist and generalist herbivores (Ali & Agrawal, 2012). In this study, we focus on the relationships between host plant defensive traits and the composition, population density, and host specificity of their leaf-chewing insects, ecologically one of the key herbivore guilds (Schoonhoven et al., 2005).

Biological interactions, including herbivory, are geographically variable; herbivore specialization varies with latitude and plants may exhibit different defensive traits when exposed to different pools of herbivores (Dyer et al., 2007; Christensen et al., 2014). Filtering geographical variation also minimizes differences in temperature, rainfall, and abundance of natural enemies, which all drive insect abundance (Connahs et al., 2011; Kozlov et al., 2013). The interplay of individual defense traits and their impact on herbivores can thus be best understood by studying co-occurring species from plant lineages with diverse chemical and morphological modes of protection. The genus *Salix*, a species-rich lineage with numerous shrub and tree species often occurring sympatrically, is an excellent model for such studies. Some species of the genus are protected by trichomes and tough leaves, which restrict herbivores from feeding and erode their mandibular jaws (Raupp, 1985; Zvereva et al., 1998), as well as by various secondary metabolites such as salicylates, flavonoids, and condensed tannins.

Salicylates are characteristic secondary metabolites of the Salicaceae; they are a family of compounds derived from salicyl alcohol and reach their highest diversity in the Salicaceae. As well as flavonoids and condensed tannins, salicylates have been repeatedly reported to have a detrimental impact on insect herbivores (Matsuki & Maclean, 1994; Kopper et al., 2002; Pearse, 2011). The anti-herbivorous function of salicylates is well recognized and their reported impacts on generalist herbivores include deterrent effects, retarded larval growth, and increased mortality (Matsuki & Maclean, 1994; Kolehmainen et al., 1995). Nevertheless, the distribution of salicylates among willows is not equal and it is well established that tissues of some willow species contain very low or zero concentrations of these secondary metabolites (Julkunen-Tiitto, 1989; Nyman & Julkunen-Tiitto, 2005). As the reduction in secondary metabolites can be a result of ineffectiveness against herbivores, it is remarkable that despite the anti-herbivorous effects of salicylates, certain specialist herbivores are known to be able to sequester salicylates and use them for protection against predators (Pasteels et al., 1983; Denno et al., 1990).

Here, we examine the defensive trait pattern of co-occurring willow species and the impact of these traits on associated leaf-chewing herbivores. First, we test whether any of the studied defensive traits are correlated and thus form distinct defense syndromes. Second, we test effectiveness of the salicylates and other defensive traits against herbivores, as indicated by their impact on herbivore abundance, species richness, and specialization. Third, we examine whether the presence, high content, and high diversity of salicylates are ancestral or derived characters among willows to explore the processes of origin and loss of host plant defensive traits.

Materials and methods

Host plants

The study was carried out within a 10 × 10 km area in South Bohemia, Czech Republic (48°51′58″–48°59′45″N, 14°26′20″–14°35′48″E) representing lowland wet meadows. This approach allowed sampling from an area with as far as possible similar abiotic conditions and with all host plants potentially available for colonization from the same pool of herbivore species.

The insects were sampled on eight willow species (out of nine growing in the area), two of their hybrids, and *Populus tremula* L., a related species of *Salix* spec. (Salicaceae) (Table 1). Shaded plants were excluded, as their traits and leaf chemistry could be significantly different. We avoided immature plants and plants that had obviously experienced browsing by herbivores or damage from other sources prior to the sampling, as these factors can cause significant changes in plant traits due to induced defense (Nakamura et al., 2005). All defensive traits were measured for 2–7 plant individuals per species (a total of 48 plants) and means were used as estimates for each species. Only two individuals of *Salix rosmarinifolia* L. and *S. viminalis* × *purpurea* were used for measuring plant traits, as other individual plants growing at our field sites were probably their clones and thus including them into analysis would not have provided additional information on trait variability. Two additional Central European lowland species (*Salix myrsinifolia* L. and *Salix alba* L.) were included in the phylogenetic analysis to make it more robust and the evolutionary trends of defensive traits more informative. Taxonomically, the studied willow species represent two subgenera of the genus *Salix*, viz., *Vetrix* and *Salix* sensu stricto (Skvortsov, 1968).

Leaf morphology

Trichome density and specific leaf area (SLA), a surrogate for leaf thickness and toughness (Groom & Lamont, 1999), were measured as parameters of leaf morphology

with possible impact on leaf-chewing insects. Trichome density was estimated as average trichome coverage (%) per 5 mm^2 area of mature leaf surface and values for dorsal and ventral side were combined.

Leaf disks of known diameter (not containing central vein) were cut and dried to a constant weight and the SLA was calculated as weight per area of the dried leaf disk. Leaf disks were sampled 10×, at 14-day intervals throughout the whole 2010 vegetative season. In total, 30 leaf disks were obtained from each plant individual.

Chemical analysis

Samples for chemical analysis were dried immediately after collection and kept in silica gel. The content (mg g^{-1}) of salicylates, flavonoids, and condensed tannins was analyzed from 5 to 9 mg of young leaves (avoiding primary and secondary leaf veins) sampled in early June. We used samples obtained in early June for the analysis of defensive trait impact on herbivores as salicylate and flavonoid concentration and diversity in young leaves tend to be higher than in leaves obtained in summer. Nevertheless, we also measured samples obtained at the beginning of August to estimate seasonal variability.

Phenolic compounds were extracted with methanol as described in Nybakken et al. (2012). Extracts were dried and kept in a freezer at −20 °C. Before the analysis, dried samples were re-dissolved in 600 μl methanol:water (1:1). We used 20 μl of re-dissolved samples for high-performance liquid chromatography (HPLC) analysis of salicylates and flavonoids following Nybakken et al. (2012). Compounds were separated using a Zorbax SBC18 (4.6 × 60 mm) HPLC column (Agilent Technologies, Waldbronn, Germany) employing a water/methanol gradient (Julkunen-Tiitto & Sorsa, 2001). Salicylate and flavonoid content was measured based on the absorbance at 220 and 320 nm, respectively. Retention times and spectra compared with those of standards were used to identify the compounds.

Soluble condensed tannins were measured using an acid-butanol assay starting with an aliquot of the HPLC sample and following the methods of Hagerman (2002). Insoluble condensed tannins were measured from tissue residues dried at room temperature. After hydrolysis, absorbance values at 550 nm were measured (Spectronic 20 Genesys spectrophotometer; Thermo Fisher Scientific, Waltham, MA, USA). The condensed tannin content was calculated based on equivalents of *Betula nana* L. leaf tannins.

Limited sampling can lead to underestimation of secondary metabolite diversity. Therefore, we reconstructed secondary metabolite accumulation curves for two willow species (*Salix cinerea* L. and *Salix fragilis* L., representing low- and high salicylate willow lineages) well represented in our sampling to estimate number of plant individuals needed for reliable secondary metabolite diversity analysis. The accumulation curves were based on Mao Tau index for the number of individuals, computed in EstimateS 8.2 (Colwell, 2006).

Host plant phylogeny reconstruction

Three loci were used for host plant phylogeny reconstruction: ITS, trnT-trnL, and ADH. Standard procedures for DNA extraction and PCR amplification with reaction conditions and primer sequences identical to those used in the original studies employing these markers were used (Taberlet et al., 1991; Cronn et al., 2002; Savage & Cavender-Bares, 2012). As multiple copies of ADH were present in each individual except *Salix viminalis* L., the ADH PCR products were cloned to separate potential paralogs and hybrid sequences. *Populus tremula* partial ADH gene sequence, accession number AJ842900 (Ingvarsson, 2005), was downloaded from GenBank.

A proportion of *S. alba*, *S. cinerea*, *S. fragilis*, and *S. myrsinifolia* individuals exhibited hybrid origin of some of their ADH sequences. This trend was pronounced in individuals growing on the same site as their sibling species. Their sequences therefore did not form monophyletic lineages and the position of a proportion of them was reconstructed with high support as an internal group within the sibling species. As these species are known to frequently hybridize with their sibling species (Skvortsov, 1968), these sequences were considered of hybrid origin and such individuals were removed from analysis.

Sequences were assembled and edited using Geneious 5.4 (Drummond et al., 2011). Trees for individual genes were not in conflict, allowing us to reconstruct the host plant phylogeny based on a matrix with all examined loci combined. Host plant phylogeny was reconstructed using the Bayesian inference in MrBayes 3.1.2 (Ronquist & Huelsenbeck, 2003). The generalized time reversible substitution model (GTR) selected using Akaike's information criterion (AIC) was used for Bayesian analysis with a flat Dirichlet prior probability density for the distribution of substitution rates and stationary nucleotide frequencies. Sampling was carried out every 10^3 generations for 10^7 generations, the first 25% of all generations were discarded as 'burnin' and the results were summarized with a 50% majority-rule consensus tree.

Insect sampling

In this study, we focused on leaf-chewing insects as one of the herbivore guilds causing the highest damage to willows. Sampling herbivores from one guild minimized the differences in feeding of examined herbivores, making the

results for different herbivores comparable. All adult and larval leaf-chewing insects were sampled during the 2008–2011 growing seasons, from the end of April to the end of September, at ca. 1-week intervals from the same tree individuals used for analysis of defense traits and their nearby conspecifics growing at the same locality (ca. 100 plants in total). Insects were sampled by sweeping and manually searching the foliage for free feeding as well as semi-concealed herbivores (leaf-tiers and leaf-rollers). Immature stages were reared to adults for identification. Dead larvae were morphotyped based on photographs, or discarded in cases when safe morphotyping proved to be impossible.

The sampling effort was equal for all plant individuals, represented by inspections that consisted of 3 min of sweeping and 3 min of manual searching. The sampling effort for different species was not completely balanced due to variation in willow population densities. For most species, the total sampling effort was 200–400 min; however, for the rare species *Salix pentandra* L. and *S. viminalis* × *purpurea* it was only 100 min.

Statistical analysis

All plant species, including willow hybrids and the single poplar species, were included in the analyses of defensive trait impact on insect diversity and population density. Willow hybrids were also included in the analyses of insect specialization on willows. These analyses are not focused on host plant evolutionary history and so the hybrid host plants can be considered independent data points with different defensive and herbivore traits. Both hybrids and the poplar species were excluded from the analysis of *Salix* defensive trait correlations, as *S. alba* × *fragilis* and *S. purpurea* × *viminalis* defensive traits patterns are products of hybridization, rather than evolution. Only non-hybrid willow species containing salicylates were used in the analysis of salicylate content and salicylate diversity correlation.

Insect abundance was expressed as population density, i.e., the number of insects sampled per unit sampling time (in min). The diversity of herbivore communities on individual willow species was estimated by species accumulation curves based on Mao Tau index, computed in EstimateS 8.2 (Colwell, 2006), plotted against the number of tree inspections. The number of species found during 40 tree inspections (corresponding to the lowest number of inspections per tree species, achieved for *S. pentandra*) was used to quantitate the herbivore species diversity of each tree species.

The impact of salicylate diversity (measured as Simpson's index of individual salicylate components), salicylate concentration, condensed tannin concentration, flavonoid concentration, trichome density, and SLA on herbivorous insect diversity and population density was analyzed by linear regression, using all nine plant species and hybrids. A phylogenetic generalized least-squares (PGLS) model was employed to test this correlation within a phylogenetic context. The optimal model of evolution was selected between Brownian and Ornstein–Uhlenbeck models, using AIC. The test was performed using nlme 3.1 and ape 2.6 packages in R 2.10.1 (R Development Core Team, 2009; Pinheiro et al., 2010). Before analysis, all predictors were log-transformed to normalize their distributions.

Linear regression was used to test correlations between defensive traits within the genus *Salix*. All variables were log-transformed and all eight *Salix* species were used as individual data points. Phylogenetic generalized least-squares were employed and as optimal model of evolution

Table 1 Mean (± SE) values of defensive traits in studied willow species and hybrids (Salicaceae)

Host plant species	Salicylate group[1]	Salicylates (mg g^{-1})	Salicylate diversity (Simpson's index)	Flavonoids (mg g^{-1})	Tannins (mg g^{-1})	SLA (cm^2 g^{-1})	Trichome cover (%)
Salix (*Vetrix*) *aurita*	LS	0.0	0	29.5 ± 1.1	196.7 ± 45.0	144.8 ± 27.0	19 ± 3.0
S. (*Vetrix*) *caprea*	LS	0.0	0	10.6 ± 1.0	139.8 ± 37.7	146.3 ± 31.8	26 ± 3.5
S. (*Vetrix*) *cinerea*	LS	0.0	0	15.0 ± 2.5	160.5 ± 62.2	131.5 ± 38.9	21 ± 2.1
S. (*Salix*) *fragilis*	HS	27.8 ± 9.4	0.79 ± 0.09	25.5 ± 6.5	51.9 ± 44.1	134.8 ± 32.7	0
S. (*Salix*) *pentandra*	HS	41.8 ± 21.5	0.65 ± 0.2	60.6 ± 6.3	192.2 ± 34.7	118.5 ± 39.6	0
S. (*Vetrix*) *purpurea*	HS	164.8 ± 19.1	0.67 ± 0.06	21.3 ± 1.4	42.6 ± 59.2	141.2 ± 39.8	0
S. (*Vetrix*) *rosmarinifolia*	HS	169.0 ± 42.0	0.64 ± 0.07	20.9 ± 0.4	134.3 ± 82.9	125.3 ± 29.9	14 ± 1.9
S. (*Vetrix*) *viminalis*	LS	0.0	0	16.0 ± 3.4	138.5 ± 35.9	165.8 ± 29.8	36 ± 7.3
S. alba × *fragilis*	LS	3.4 ± 1.9	0.49 ± 0.08	27.3 ± 4	127.3	105.3	2 ± 0.8
S. viminalis × *purpurea*	LS	2.6 ± 1.3	0	33.1 ± 3.8	112.3	160.4	0
Populus tremula L.	–	19.4 ± 20.7	0.34 ± 0.32	33.8 ± 5.9	37.9 ± 35.0	144.5 ± 76.9	0

SLA, specific leaf area.

[1]'LS' and 'HS' categories indicate willows with, respectively, low and high salicylate concentration and diversity.

was selected as above to test for correlation between defensive traits within a phylogenetic context.

In the analysis of salicylate impact on insect specialization, we divided willows into two groups – species with high salicylate diversity and concentration (high salicylate, 'HS' species) and species with low salicylate diversity and concentration (low salicylate, 'LS' species; Table 1). We used three host specificity indices, each measuring a different aspect of insect specialization. (1) The proportion of Salicaceae specialists, i.e., species feeding only on Salicaceae (based on Smreczyński, 1966, 1972; Lacourt, 1999; Warchalowski, 2003; Macek et al., 2007, 2008, 2012), was estimated for each *Salix* species and compared between those with low and high salicylate content by ANOVA with arc-sin data transformation. (2) Herbivore specialization on plant species containing salicylates was estimated for each herbivore as the Salicilate Specificity Index (SSI), measuring its distribution between high (HS) and low (LS) salicylate species as follows: mean density per HS species/(mean density per HS species + mean density per LS species) (see Table 1). The SSI values range from 1 for complete HS specialists, through 0.5 for herbivores indifferent to salicylate content, to 0 for complete LS specialists. The mean SSI values of their individual herbivores were compared between HS and LS willow species. (3) Host-range breadth within the Salicaceae was based on our sampling of herbivores. It was measured quantitatively using Simpson's index, capturing the density distribution for each herbivore species among the studied willow species. The herbivore community on each willow species was characterized by the mean host-range breadth, calculated as average value of host-range breadth for all its constituent species. Resulting values of specialization were compared between communities on willows with high and low salicylate content by ANOVA with arcsine data transformation applied to frequency values. Singletons and doubletons were excluded as uninformative from all host specificity analyses.

Results

Host plant phylogeny and defensive traits

The phylogram reconstructed based on Bayesian inference suggests monophyly of both examined willow subgenera, *Salix* and *Vetrix* (Figure 1). However, support for some clades is low, which complicates our interpretation of how defensive traits might have evolved. The most ambiguous is the position of *S. viminalis*, which often forms a monophyletic group with *Salix purpurea* L. and *S. rosmarinifolia*.

There was large interspecific variability in willow defensive traits (Table 1). Flavonoids and condensed tannins were found in leaves of all studied host plants, and the content of salicylates varied from 0 to 22% of leaf dry mass in young leaves among species (Figures 1A and S1, Table S1). The highest salicylate content and diversity was found in the leaves of rather basal *S. rosmarinifolia* and *S. purpurea*. Moderate diversity and content was found in *S. fragilis*, *S. pentandra*, and *P. tremula*, suggesting with high support at least two independent losses of salicylates (Figure 1B, Table 1).

In total, 108 flavonoid and 28 salicylate compounds and their derivatives were found (Table S1). Flavonoids and salicylates exhibited a high proportion of species-specific compounds, 56 and 46%, respectively (Figure S1). No compound was shared by all willow species. We observed

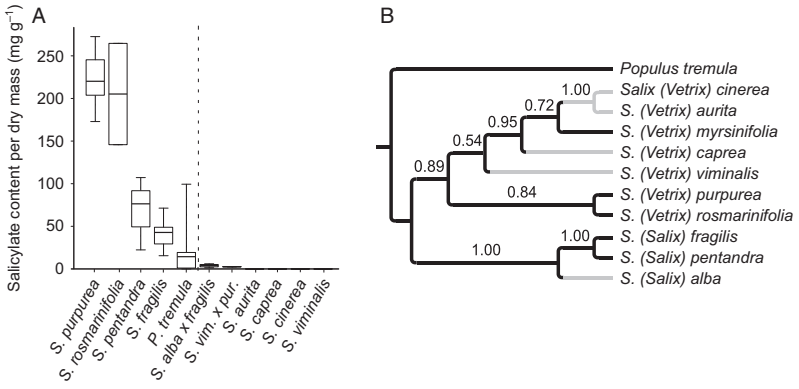

Figure 1 Salicylate content in studied *Salix* and *Populus* host plant species and hybrids and the distribution of salicylates throughout the willow phylogeny. (A) Salicylate content in young leaves. The boxes indicate the first to third quartiles with the medians as thick horizontal lines, the whiskers indicate ranges. The dashed line separates the five species with high salicylate content (on the left) from species with low salicylate content. (B) Phylogeny as reconstructed by Bayesian inference. The support of clades is characterized by posterior probabilities. Grey indicates lineages with low salicylate content, as estimated from our measurements and the literature (Julkunen-Tiitto, 1989).

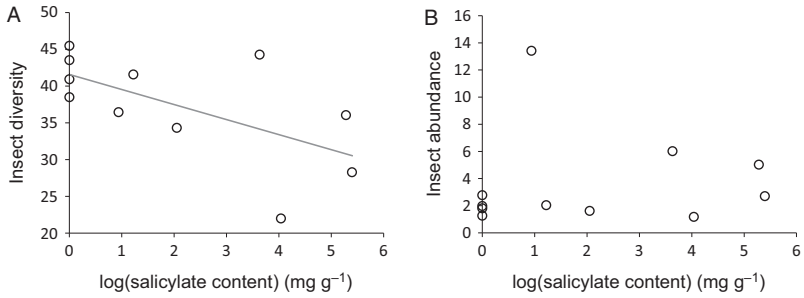

Figure 2 Impact of salicylates on insect communities. The correlation of salicylate content (mg g^{-1}) with the (A) diversity ($F_{2,9}$ = 7.37, P = 0.043) and (B) density ($F_{2,9}$ = 0.03, P = 0.87) of leaf-chewing insects. As measure of insect density was taken the number of insect individuals divided by sampling effort, expressed as the time (min) used for sampling insects. The diversity of herbivore communities was estimated using species accumulation curves based on Mao Tau index.

an increase in salicylates and flavonoids and a decrease in condensed tannins during the season. Nevertheless, the difference was quantitative rather than qualitative and the relative differences between species remained very similar (Table S2). The accumulation curves revealed that secondary metabolite analysis of a relatively low number of willow individuals is necessary to reach a plateau of diversity of willow secondary metabolites, making the estimates based even on only three plant individuals satisfactory (Figure S2).

Defensive traits were not correlated between plant species (Table S3) except for a negative correlation between salicylate content and trichome density ($F_{1,6}$ = 5.52, P = 0.043 for linear regression, $F_{1,6}$ = 4.98, P = 0.035 for PGLS) and salicylate diversity and trichome density ($F_{1,6}$ = 20.29, P = 0.004 for linear regression, $F_{1,6}$ = 18.36, P = 0.003 for PGLS).

Herbivore diversity and population density

We collected 9 196 individuals from 201 species of leaf-chewing insects, representing adult beetles and their larvae, caterpillars, and sawfly larvae from a total of 21 families (Table 2). From the host plant defense traits studied, only salicylate content had a significant negative impact on herbivore diversity ($F_{2,9}$ = 7.37, P = 0.043 for linear regression, $F_{2,9}$ = 7.98, P = 0.020 for PGLS; Figure 2, Table S4), whereas the impact of salicylate diversity (measured as Simpson's index of individual salicylate components) was not significant ($F_{2,9}$ = 2.46, P = 0.15 for linear regression, $F_{2,9}$ = 1.74, P = 0.23 for PGLS). Neither salicylate content nor any other defensive trait exhibited a significant effect on overall insect herbivore density (Figure 2, Table S4). We carried out separate analyses of effect on insect density for all 28 salicylates and their derivatives, but none of the examined compounds exhibited a significant or marginally significant impact on herbivore density or diversity.

Insect specialization

We found salicylates to influence insect specialization. Communities harbored by willows with high salicylate content exhibited a higher ratio of Salicaceae specialists to generalists (Figure 3). On high-salicylate willows, herbivores were to some extent specialized on these high-salicy-

Table 2 The total number of species/Salicaceae-specialist species (feeding only on Salicaceae) sampled from insect herbivore families

Lepidoptera		Coleoptera		Hymenoptera	
Arctiidae	3/0	Attelabidae	3/1	Argidae	2/1
Depressariidae	2/2	Cerambycidae	2/0	Tenthredinidae	52/49
Drepanidae	1/1	Chrysomelidae	24/12		
Gelechiidae	2/2	Curculionidae	30/17		
Geometridae	27/6	Scarabaeidae	1/0		
Lymantridae	4/0	Tenebrionidae	1/0		
Noctuidae	25/6				
Nolidae	2/1				
Notodontidae	8/4				
Pyralidae	1/1				
Saturniidae	1/1				
Sphingidae	1/1				
Tortricidae	7/2				

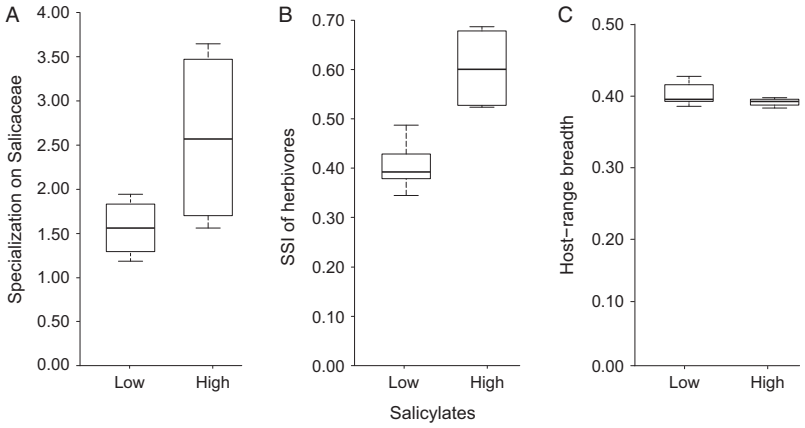

Figure 3 Impact of salicylates on insect specialization. (A) Ratio of Salicaceae specialist-to-generalist species, (B) SSI (i.e., Salicylate Specificity Index, indicating specialization on willow species containing salicylates), and (C) host specificity of herbivore species (host-range breadth) on willows with high and low salicylate content. Ratio of specialist-to-generalist species (A) and SSI (B) were significantly different on host species with high vs. low salicylate content ($F_{1,7} = 7.08$, $P = 0.029$ and $F_{1,7} = 72.14$, $P = 0.002$, respectively), whereas there was no difference in host-range breadth (C; $F_{1,7} = 1.54$, $P = 0.25$). The boxes indicate the first to third quartiles with the medians as thick horizontal lines, the whiskers indicate ranges.

late hosts (SSI>0.5), and vice versa, the herbivore species on low salicylate willows were specialized on low-salicylate hosts (SSI<0.5; Figure 3). In either case, this specialization was not absolute, as indicated by the SSI values $\gg 0$ and $\ll 1$, respectively. Separate analyses for Coleoptera, Lepidoptera, and Hymenoptera produced a similar trend of SSI (Figure S3). On the other hand, the host specificity of herbivore species within the examined set of host plants did not differ between herbivore communities from willows with low and high salicylate content (Figure 3).

Within the studied set of eight *Salix* species, generalist herbivores used the same number of host species as Salicaceae specialists ($F_{1,7} = 2.16$, $P = 0.15$). However, we found major differences between Coleoptera, Lepidoptera, and Hymenoptera when insect lineages were analyzed separately. Salicaceae specialists used significantly more host species than generalists within both Coleoptera and Lepidoptera (Figure S4), whereas the generalists were almost entirely lacking in Hymenoptera (Table 2).

Discussion

Salicylates, which represent a group of secondary metabolites unique for Salicaceae, had the most pronounced impact of all examined defensive traits, affecting diversity of leaf-chewing herbivores and some characteristics of host specialization. Communities on willows with high salicylate content were most specialized, with the majority of herbivores being known to feed solely on the Salicaceae family and preferring a diet containing salicylates. The high number of species feeding only on Salicaceae suggests that a certain level of specialization is needed to overcome high salicylate content.

Salicylates have been reported to have various negative effects on generalist herbivores (Matsuki & Maclean, 1994; Rank et al., 1998). However, only a few previous studies attempted to examine the effect of willow salicylates at insect community level (Topp et al., 2002). We demonstrate that high salicylate content causes partial exclusion of generalist herbivores, resulting in less diverse communities on salicylate-rich hosts. Salicylates therefore pose an effective feeding barrier for many generalist species otherwise common on willows lacking these secondary metabolites.

None of the other examined defensive traits had a significant impact on leaf-chewing insects, although their detrimental effect on certain insect species' feeding or survival has been repeatedly recorded (Raupp, 1985; Zvereva et al., 1998; Kopper et al., 2002; Lahtinen et al., 2006). Similar situations in which recognized defensive traits, such as trichomes or latex outflow, do not affect insect population densities have been recorded before (Basset & Novotny, 1999). The lack of any impact may indicate that certain herbivores are able to cope with specific defensive traits and hence compensate for partial exclusion of non-adapted species under field conditions.

Salicylates thus play a major role in forming insect communities on willows. Pronounced impact of salicylates, especially when compared with the effect of other examined defensive traits, implies that some insect species have

not been able to adapt to salicylates. This could be due to the scarce distribution of salicylates among plants, which is in congruence with the biochemical barrier theory (Jones & Lawton, 1991), suggesting that generalists are excluded from insect communities harbored by plants with unique or highly toxic secondary metabolites, as found by previous studies (Becerra, 1997; Agrawal, 2005).

Salicylate-rich willows harbored a higher proportion of specialists feeding only on the Salicaceae family, showing that high salicylate content narrows the total host range of associated herbivores. On the other hand, salicylates did not have any negative effect on insect relative host ranges within the examined set of willow species. The willows with high salicylate content thus harbored insects feeding on the same proportion of examined willow species as their salicylate-poor relatives. Moreover, Salicaceae specialists in both Coleoptera and Lepidoptera used more willow species, within the examined set of willows, than herbivores feeding also on other families. This implies that Salicaceae specialists are better at using a variety of willows, whereas generalists may be confined only to particular willow species.

Total insect density was unaffected by salicylate content and diversity. Specialist willow herbivores have even been reported to benefit from salicylates, using them for the production of defensive compounds (Pasteels et al., 1983; Denno et al., 1990) or possibly as a source of glucose (Rowell-Rahier & Pasteels, 1986; Rank et al., 1998). These specialists can thus reach very high population densities on willows with high salicylate content. They can be much more abundant on high salicylate species than generalist insect herbivores on willows with no salicylates. This would explain the observed situation in which there is no significant impact of salicylates on the total insect abundance.

The low protective value of salicylates against specialized insects is also suggested by other local studies throughout Europe and North America, which found high population densities of specialized herbivores on willows with high salicylate content (Denno et al., 1990; Kolehmainen et al., 1995; Martinsen et al., 1998). The specialists' density appears to be driven by nitrogen content or leaf quality in such cases (Nakamura et al., 2005). Salicylates thus appear to be to a large extent ineffective against the majority of specialized herbivores associated with willows throughout their geographic ranges.

Production of salicylates requires a large investment of energy, as the total salicylate content can reach up to 22% of dry leaf mass in the early stages of leaf development. In *S. purpurea* and *S. rosmarinifolia*, the energy allocated to salicylates seems to be higher than the allocation to condensed tannins and flavonoids combined (Gershenzon, 1994). Such considerable investment has lead to a trade-off between salicylate production and plant growth (Osier & Lindroth, 2001). Although a high concentration of salicylates negatively influences communities of generalist herbivores, it has no impact or even a positive influence on specialists and no overall impact on total herbivore abundance.

Low protective value of salicylates against specialized herbivores and the high energy allocation required for their synthesis may have led to the loss of salicylates in some willow lineages. Although some willow species contain very low to zero concentrations of salicylates (Julkunen-Tiitto, 1989; Nyman & Julkunen-Tiitto, 2005), the possession of salicylates appears to be an ancestral state within the genus *Salix*, with salicylates being widespread among poplars, the sister genus of willows (Palo, 1984; Leskinen & Alstrom-Rapaport, 1999). The absence of salicylates in some of the derived lineages within the genus *Salix* thus appears to be secondary. Our interpretation of salicylate evolution within the genus *Salix* is complicated by low support for some clades and the limited extent of our dataset. Additional plant species would be required to describe salicylate evolution in willows. Although we cannot document the exact course of salicylate evolution, our results, along with previous studies documenting the lack of salicylates in many willow species (Julkunen-Tiitto, 1989; Nyman & Julkunen-Tiitto, 2005), suggest that willows lost salicylates repeatedly during their evolution – at least 2 or 3× during the evolution of the willow species we studied.

A negative correlation of content and diversity of salicylates with density of trichomes suggests that willows lacking salicylates may rely more on mechanical defenses. Although we failed to find any negative correlation between trichome density and insect population density in this study, willow trichomes have been reported to influence both Salicaceae specialists and generalists, making them potentially effective against a broad spectrum of herbivores (Matsuki & Maclean, 1994; Zvereva et al., 1998).

Moreover, divergence in defensive traits may help related species growing in sympatry to avoid herbivory. As many insect herbivores are phylogenetically conservative in their food choice (Schoonhoven et al., 2005), host-shifts among related plants with similar defense are very likely. Large interspecific differences among sibling species may make these shifts less common. Induced defense, pronounced in many willow species (e.g., Nakamura et al., 2005), which also increases variation in plant defense, may play a similar role. In turn, related species often exhibit diverging defense strategies (e.g., Agrawal & Fishbein, 2008; Fincher et al., 2008) and herbivory was reported to bias community assembly toward chemical heterogeneity

(Becerra, 2007). In willows, benefits brought by a large variation in defensive traits may have resulted in the observed situation in which some species are defended by salicylates and some by trichomes, which may be another factor driving the divergence in willow defenses and in turn enhancing the selection against salicylates in some species.

In conclusion, our results show that salicylates do not lower insect abundance in the local communities we studied. We suggest that the lack of effect on insect herbivore abundance may be one of the factors driving the loss of host plant defensive traits. In the case of willows, several lineages of highly specialized leaf-chewing herbivores were able to adapt to salicylates, making the required high energetic allocation to this defensive trait costly. Diversification of salicylates might have met a dead end and certain willow lineages may presumably use the energy saved for maintaining other strategies of defense. Although plant defenses have probably diversified during the course of plant evolution (Becerra et al., 2009), our results suggest that certain defensive traits might be lost or reduced in insect-plant systems with high insect specialization and high defense costs, as in willows or *Asclepias* spp. (Agrawal & Fishbein, 2008). These findings thus illustrate that evolution of plant defensive traits is a dynamic process rather than a simple directional trend.

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References

Agrawal AA (2005) Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. Evolutionary Ecology Research 7: 651–667.

Agrawal AA & Fishbein M (2008) Phylogenetic escalation and decline of plant defense strategies. Proceedings of the National Academy of Sciences of the USA 105: 10057–10060.

Agrawal AA, Hastings AP, Johnson MT, Maron JL & Salminen J-P (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. Science 338: 113–116.

Ali JG & Agrawal AA (2012) Specialist versus generalist insect herbivores and plant defense. Trends in Plant Science 17: 293–302.

Basset Y & Novotny V (1999) Species richness of insect herbivore communities on *Ficus* in Papua New Guinea. Biological Journal of the Linnean Society 67: 477–499.

Becerra JX (1997) Insects on plants: macroevolutionary chemical trends in host use. Science 276: 253–256.

Becerra JX (2007) The impact of herbivore-plant coevolution on plant community structure. Proceedings of the National Academy of Sciences of the USA 104: 7483–7488.

Becerra JX, Noge K & Venable DL (2009) Macroevolutionary chemical escalation in an ancient plant-herbivore arms race. Proceedings of the National Academy of Sciences of the USA 106: 18062–18066.

Benderoth M, Textor S, Windsor AJ, Mitchell-Olds T, Gershenzon J & Kroymann J (2006) Positive selection driving diversification in plant secondary metabolism. Proceedings of the National Academy of Sciences of the USA 103: 9118–9123.

Christensen S, Heimes C, Agerbirk N, Kuzina V, Olsen CE & Hauser TP (2014) Different geographical distributions of two chemotypes of *Barbarea vulgaris* that differ in resistance to insects and a pathogen. Journal of Chemical Ecology 40: 491–501.

Coley PD, Lokvam J, Rudolph K, Bromberg K, Sackett TE et al. (2005) Divergent defensive strategies of young leaves in two species of *Inga*. Ecology 86: 2633–2643.

Colwell RK (2006) Statistical Estimation of Species Richness and Shared Species from Samples, version 8. Available at: http://wwwpurtloclcorg/estimates.

Connahs H, Aiello A, Van Bael S & Rodriguez-Castaneda G (2011) Caterpillar abundance and parasitism in a seasonally dry versus wet tropical forest of Panama. Journal of Tropical Ecology 27: 51–58.

Cronn RC, Small RL, Haselkorn T & Wendel JF (2002) Rapid diversification of the cotton genus (*Gossypium*: Malvaceae) revealed by analysis of sixteen nuclear and chloroplast genes. American Journal of Botany 89: 707–725.

Denno RF, Larsson S & Olmstead KL (1990) Role of enemy-free space and plant quality in host-plant selection by willow beetles. Ecology 71: 124–137.

Drummond AJ, Ashton B, Buxton S, Cheung M, Cooper A et al. (2011) Geneious v54. Available at: http://www.geneious.com.

Dyer LA, Singer MS, Lill JT, Stireman JO, Gentry GL et al. (2007) Host specificity of Lepidoptera in tropical and temperate forests. Nature 448: 696–699.

Ehrlich PR & Raven PH (1964) Butterflies and plants – a study in coevolution. Evolution 18: 586–608.

Fincher RM, Dyer LA, Dodson CD, Richards JL, Tobler MA et al. (2008) Inter- and intraspecific comparisons of antiherbivore

defenses in three species of rainforest understory shrubs. Journal of Chemical Ecology 34: 558–574.
Fucile G, Falconer S & Christendat D (2008) Evolutionary diversification of plant shikimate kinase gene duplicates. PLoS Genetics 4: e1000292.
Gershenzon J (1994) The cost of plant chemical defense against herbivory: a biochemical perspective. Insect-Plant Interactions, Vol. 5 (ed. by EA Bernays), pp. 105–173. CRC Press, Boca Raton, FL, USA.
Groom PK & Lamont BB (1999) Which common indices of sclerophylly best reflect differences in leaf structure? Ecoscience 6: 471–474.
Hagerman AE (2002) The Tannin Handbook. Miami University, Oxford, OH, USA.
Ingvarsson PK (2005) Nucleotide polymorphism and linkage disequilbrium within and among natural populations of European Aspen (*Populus tremula* L., Salicaceae). Genetics 169: 945–953.
Jones CG & Lawton JH (1991) Plant chemistry and insect species richness of British umbellifers. Journal of Animal Ecology 60: 767–777.
Julkunen-Tiitto R (1989) Phenolic constituents of *Salix* – a chemotaxonomic survey of further Finnish species. Phytochemistry 28: 2115–2125.
Julkunen-Tiitto R & Sorsa S (2001) Testing the effects of drying methods on willow flavonoids, tannins, and salicylates. Journal of Chemical Ecology 27: 779–789.
Kliebenstein DJ & Osbourn A (2012) Making new molecules – evolution of pathways for novel metabolites in plants. Current Opinion in Plant Biology 15: 415–423.
Kolehmainen J, Julkunen-Tiitto R, Roininen H & Tahvanainen J (1995) Phenolic glucosides as feeding cues for willow-feeding leaf beetles. Entomologia Experimentalis et Applicata 74: 235–243.
Kopper BJ, Jakobi VN, Osier TL & Lindroth RL (2002) Effects of paper birch condensed tannin on whitemarked tussock moth (Lepidoptera: Lymantriidae) performance. Environmental Entomology 31: 10–14.
Kozlov MV, van Nieukerken EJ, Zverev V & Zvereva EL (2013) Abundance and diversity of birch-feeding leafminers along latitudinal gradients in northern Europe. Ecography 36: 1138–1149.
Kursar TA, Dexter KG, Lokvam J, Pennington RT, Richardson JE et al. (2009) The evolution of antiherbivore defenses and their contribution to species coexistence in the tropical tree genus *Inga*. Proceedings of the National Academy of Sciences of the USA 106: 18073–18078.
Lacourt J (1999) Répertoire des Tenthredinidae Ouest-Paléarctiques (Hymenoptera, Symphyta). Société Entomologique de France, Paris, France.
Lahtinen M, Kapari L, Haukioja E & Pihlaja K (2006) Effects of increased content of leaf surface flavonoids on the performance of mountain birch feeding sawflies vary for early and late season species. Chemoecology 16: 159–167.
Leskinen E & Alstrom-Rapaport C (1999) Molecular phylogeny of Salicaceae and closely related Flacourtiaceae: evidence from 5.8 S, ITS 1 and ITS 2 of the rDNA. Plant Systematics and Evolution 215: 209–227.
Macek J, Dvorak J, Traxler L & Cervenka V (2007) Noční Motýli I. Academia, Prague, Czech Republic.
Macek J, Dvorak J, Traxler L & Cervenka V (2008) Noční Motýli II. Academia, Prague, Czech Republic.
Macek J, Prochazka J & Traxler L (2012) Noční Motýli III. Academia, Prague, Czech Republic.
Martinsen GD, Driebe EM & Whitham TG (1998) Indirect interactions mediated by changing plant chemistry: beaver browsing benefits beetles. Ecology 79: 192–200.
Matsuki M & Maclean SF (1994) Effects of different leaf traits on growth rates of insect herbivores on willows. Oecologia 100: 141–152.
Nakamura M, Utsumi S, Miki T & Ohgushi T (2005) Flood initiates bottom-up cascades in a tri-trophic system: host plant regrowth increases densities of a leaf beetle and its predators. Journal of Animal Ecology 74: 683–691.
Nybakken L, Horkka R & Julkunen-Tiitto R (2012) Combined enhancements of temperature and UVB influence growth and phenolics in clones of the sexually dimorphic *Salix myrsinifolia*. Physiologia Plantarum 145: 551–564.
Nyman T & Julkunen-Tiitto R (2005) Chemical variation within and among six northern willow species. Phytochemistry 66: 2836–2843.
Osier TL & Lindroth RL (2001) Effects of genotype, nutrient availability, and defoliation on aspen phytochemistry and insect performance. Journal of Chemical Ecology 27: 1289–1313.
Palo RT (1984) Distribution of birch (*Betula* spp.), willow (*Salix* spp.), and poplar (*Populus* spp.) secondary metabolites and their potential role as chemical defense against herbivores. Journal of Chemical Ecology 10: 499–520.
Pasteels JM, Rowell-Rahier M, Braekman JC & Dupont A (1983) Salicin from host plant as precursor of salicylaldehyde in defensive secretion of chrysomeline larvae. Physiological Entomology 8: 307–314.
Pearse IS (2011) The role of leaf defensive traits in oaks on the preference and performance of a polyphagous herbivore, *Orgyia vetusta*. Ecological Entomology 36: 635–642.
Pinheiro J, Bates D, DebRoy S, Sarkar D & Team RDC (2010) nlme: Linear and Nonlinear Mixed Effects Models, R package version 3.1-97. R Foundation for Statistical Computing, Vienna, Austria.
R Development Core Team (2009) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria.
Rank NE, Kopf A, Julkunen-Tiitto R & Tahvanainen J (1998) Host preference and larval performance of the salicylate-using leaf beetle *Phratora vitellinae*. Ecology 79: 618–631.
Raupp MJ (1985) Effects of leaf toughness on mandibular wear of the leaf beetle, *Plagiodera versicolora*. Ecological Entomology 10: 73–79.

Ronquist F & Huelsenbeck JP (2003) MRBAYES 3: Bayesian phylogenetic inference under mixed models. Bioinformatics 19: 1572–1574.

Rowell-Rahier M & Pasteels JM (1986) Economics of chemical defense in Chrysomelinae. Journal of Chemical Ecology 12: 1189–1203.

Savage JA & Cavender-Bares J (2012) Habitat specialization and the role of trait lability in structuring diverse willow (genus *Salix*) communities. Ecology 93: S138–S150.

Schoonhoven LM, van Loon JJA & Dicke M (2005) Insect-Plant Biology, 2nd edn. Oxford University Press, Oxford, UK.

Skvortsov AK (1968) Willows of the USSR: A Taxonomic and Geographic Revision. Nauka, Moscow, Russia.

Smreczyński S (1966) Klucze do Oznaczania Owadów Polski XIX: Chrząszcze – Coleoptera Zeszyt 98 b, Ryjkowce – Curculionidae, Podrodziny Otiorhynchinae, Brachyderinae. Państvove Wydawnictwo Naukove, Warsaw, Poland.

Smreczyński S (1972) Klucze do Oznaczania Owadów Polski XIX: Chrzaszcze – Coleoptera. Zeszyt 98 d, Ryjkowce – Curculionidae, Podrodzina Curculioninae, Plemiona Dryophthorini, Cossonini, Bagoini, Tanysphyrini, Notarii, Smicronychini, Ellescini, Acaliptini, Tychiini, Anthonomini, Curculiononi, Pissodini, Magdalini, Trachodini, Rhynchophorini, Cryptorhynchini. Panstvove Wydawnictwo Naukove, Warsaw, Poland.

Taberlet P, Gielly L, Pautou G & Bouvet J (1991) Universal primers for amplification of three noncoding regions of chloroplast DNA. Plant Molecular Biology 17: 1105–1109.

Topp W, Kulfan J, Zach P & Nicolini F (2002) Beetle assemblages on willow trees: do phenolic glycosides matter? Diversity and Distributions 8: 85–106.

Warchalowski A (2003) Chrysomelidae: The Leaf Beetles of Europe and the Mediterranean Area. Natura Optima Dux Foundation, Warsaw, Poland.

Zvereva EL, Kozlov MV & Niemela P (1998) Effects of leaf pubescence in *Salix borealis* on host-plant choice and feeding behaviour of the leaf beetle *Melasoma lapponica*. Entomologia Experimentalis et Applicata 89: 297–303.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Number of flavonoids and salicylates found in examined *Salix* and *Populus* host plants. Grey indicates species-unique compounds.

Figure S2. Secondary metabolite diversity accumulation curves for *Salix cinerea* (black) and *Salix fragilis* (gray) based on Mao Tau index plotted against number of individuals analyzed.

Figure S3. Specialization on diet containing salicylates by examined insect taxa on willows with high and low salicylate content. (A) Coleoptera ($F_{1,41} = 32.39$, $P<0.001$), (B) Lepidoptera ($F_{1,39} = 11.30$, $P = 0.010$), and (C) Hymenoptera ($F_{1,32} = 15.79$, $P = 0.004$). The boxes indicate the first to third quartiles with the median as thick horizontal lines, the whiskers indicate ranges.

Figure S4. Number of willow host plant species used by Salicaceae specialists and generalists. There was no difference in the number of willow species used between Salicaceae specialists and generalists ($F_{1,114} = 2.02$, $P = 0.16$), but separate analyses for Coleoptera and Lepidoptera revealed more willow hosts in Salicaceae specialists than in generalists (Coleoptera: $F_{1,41} = 7.699$, $P<0.01$; Lepidoptera: $F_{1,39} = 6.066$, $P = 0.018$). Hymenoptera, including highly specialized sawflies, exhibited the narrowest host spectra. For Hymenoptera the specialist/generalist spectrum breadth difference was not analyzed, as there was only one generalist species with more than two individuals present in our samples. The boxes indicate the first to third quartiles with the medians as thick horizontal lines, the whiskers indicate ranges.

Table S1. Mean concentrations (mg g^{-1}) of secondary metabolites found in examined *Salix* and *Populus* host plant species and hybrids.

Table S2. Seasonal variability in secondary metabolite content (mg g^{-1}) and salicylate diversity (Simpson's index). The samples for analysis were obtained in early June ('spring') and early August ('summer').

Table S3. Correlation between defensive traits of studied willow species ($F_{1,6}$/P values, based on PGLS and linear regression). The lower triangle shows values obtained from analysis using phylogenetic generalized least squares, the upper triangle shows values obtained from simple linear regression.

Table S4. Impact of *Salix* defensive traits on leaf-chewing insect diversity and density. Table includes $F_{2,9}$/P-values, based on simple linear regression (Linear) and phylogenetic generalized least squares (PGLS).

Supporting Information

Insect herbivores drive the loss of unique chemical defense in willows

Martin Volf, Riitta Julkunen-Tiitto, Jan Hrcek & Vojtech Novotny

Figure S1**.** Number of salicylates and flavonoids found in examined host-plants. Grey color indicates species-unique compounds.

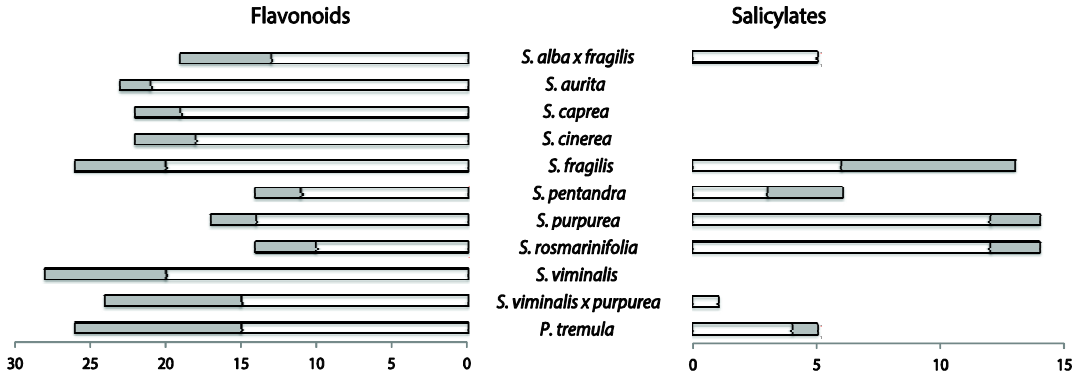

Figure S2**.** Secondary metabolite diversity accumulation curves for *Salix cinerea* (black) and *S. fragilis* (grey) based on Mao Tau index plotted against number of individuals analyzed.

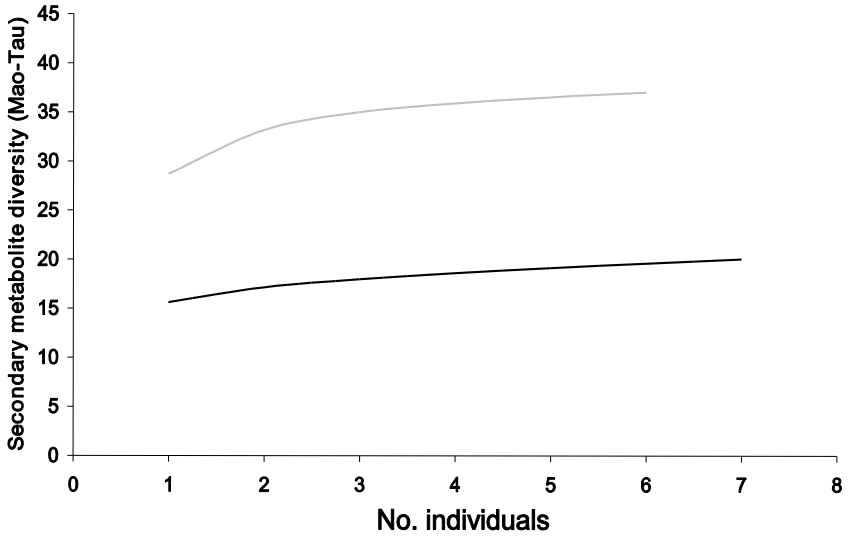

Figure S3. Specialization on diet containing salicylates by examined insect taxa on willows with high and low salicylate content. A: Coleoptera ($F_{(1,41)}$=32.39, p<0.001), B: Lepidoptera ($F_{(1,39)}$=11.30, p=0.010), C: Hymenoptera ($F_{(1,32)}$=15.79, p=0.004). The box shows the first to third quartile with the median as a horizontal line, the whiskers show range.

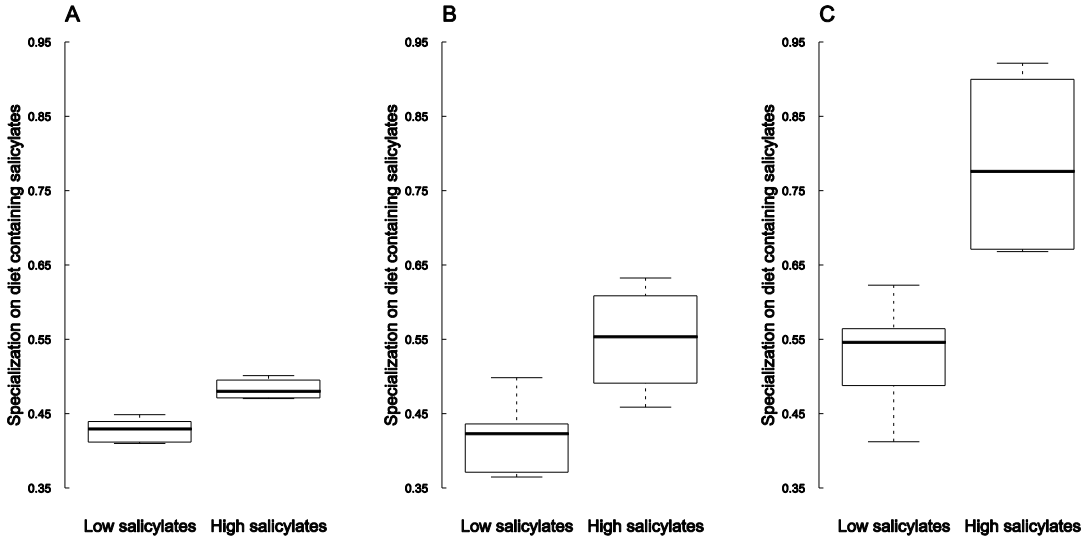

Figure S4. Number of willow host plant species used by Salicaceae specialists and generalists. There was no difference in the number of used willow species between Salicaceae specialists and generalists ($F_{(1,114)}$= 2.02, p=0.158), but separate analysis for Coleoptera and Lepidoptera revealed significantly high number of willow hosts in Salicaceae specialists than generalists (D) (Coleoptera: $F_{(1,41)}$=7.6989, p>0.01; Lepidoptera: $F_{(1,39)}$=6.066, p=0.018). Hymenoptera, including highly specialized sawflies, exhibited the narrowest host-spectra. For Hymenoptera the specialist/generalist spectra breadth difference was not analysed, since there was only one generalist species with more than two individuals present in our samples. The box shows the first to third quartile with the median as a horizontal line, the whiskers show range.

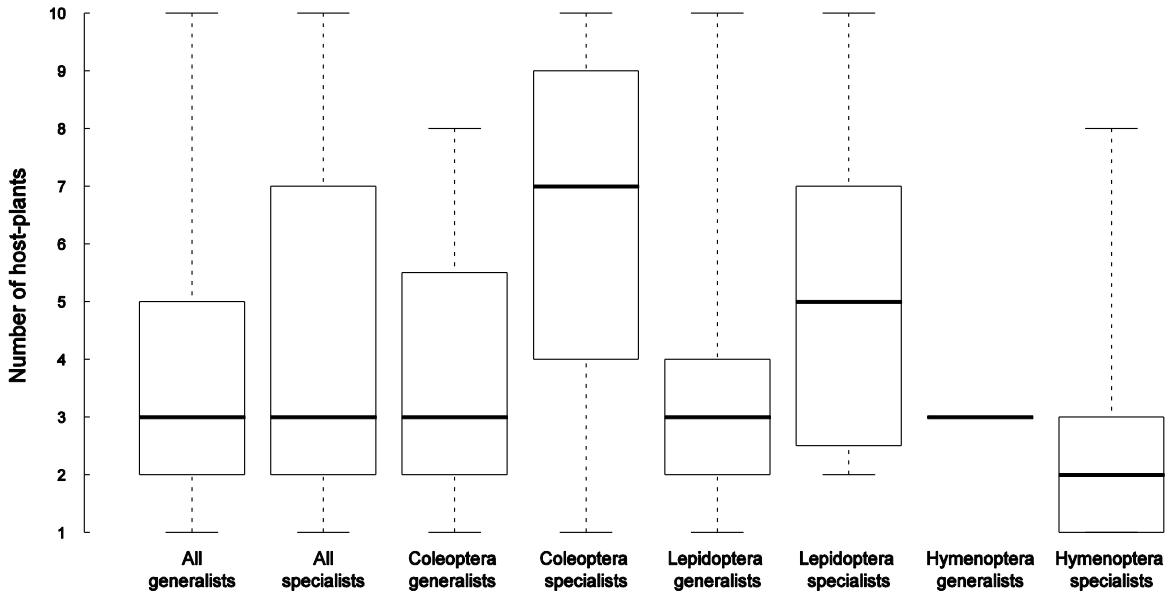

Table S1. List of secondary metabolites found in examined host-plants. Values indicate mean contrations (mg/g).

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
Salicylates											
2`-O-acetylsalicin	-	-	-	-	-	5.603	-	-	-	-	-
acetyl-salicortin	-	-	-	-	-	21.144	-	-	-	-	2.227
cinnamoyl acetyl-salicortin	-	-	-	-	-	0.341	-	-	-	-	-
cinnamoyl salicylate 1	-	-	-	-	-	-	-	4.137	-	-	-
cinnamoyl salicylate 2	-	-	-	-	-	-	1.898	-	-	-	-
cinnamoyl salicylate 3	-	-	-	-	-	-	1.457	-	-	-	-
cinnamoyl salicylate 4	-	-	-	-	-	-	-	0.081	-	-	-
cinnamoyl salicortin	-	-	-	-	-	-	-	-	-	-	0.617
cinnamoyl tremulacin	-	-	-	-	0.080	-	-	-	-	-	-
cinnamoyl tremuloidin	-	-	-	-	3.212	-	-	-	-	-	-
disalicortin	-	-	-	-	-	-	4.349	3.327	-	-	-
ditremulacin derivative 1	-	-	-	-	-	-	2.039	1.059	-	-	-
ditremulacin derivative 2	-	-	-	-	-	-	2.138	0.999	-	-	-
ditremulacin derivative 3	-	-	-	-	-	-	0.417	0.224	-	-	-
ditremulacin derivative 4	-	-	-	-	0.240	-	1.329	2.081	-	-	-
HCH-acetyl-salicortin	-	-	-	-	-	10.658	-	-	-	-	-
HCH-salicortin	-	-	-	-	0.608	-	-	-	-	-	-
HCH-tremulacin derivative 1	-	-	-	-	-	-	0.702	0.507	-	-	-
HCH-tremulacin derivative 2	-	-	-	-	-	-	0.801	0.537	-	-	-
salicin	0.680	-	-	-	1.161	3.231	13.999	15.047	-	1.636	2.847
salicortin	0.001	-	-	-	3.468	-	70.265	88.099	-	-	3.980
salicyl alcohol	-	-	-	-	0.089	-	2.165	3.763	-	-	-
salicyl alcohol-diglucoside	1.453	-	-	-	2.386	-	-	-	-	-	-
tremulacin	0.138	-	-	-	10.330	-	60.361	47.421	-	-	9.773
tremulacin derivative 1	0.001	-	-	-	0.960	-	-	-	-	-	-
tremulacin derivative 2	-	-	-	-	0.035	-	-	-	-	-	-

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
tremulacin derivative 3	-	-	-	-	0.595	0.769	1.299	1.758	-	-	-
tremuloidin	-	-	-	-	4.599	-	-	-	-	-	-
Flavonoids											
(+)-catechin	-	7.785	0.584	4.184	-	-	1.371	1.861	2.838	1.214	1.344
apigenin 5-glucoside	-	-	-	-	-	-	-	0.467	-	-	-
apigenin 7-glucoside	-	-	-	0.313	-	-	0.483	0.342	0.050	0.349	-
apigenin derivative 1	-	-	-	-	-	-	-	0.026	-	-	-
apigenin derivative 2	-	-	-	-	-	-	-	-	-	-	0.078
apigenin derivative 3	-	-	-	-	-	-	0.153	-	-	-	-
apigenin derivative 4	-	-	-	0.016	-	-	-	-	-	-	-
apigenin derivative 5	-	-	-	0.005	-	-	-	-	-	-	-
chlorogenic acid	8.132	2.605	-	1.266	8.861	23.361	-	-	0.736	-	2.459
chlorogenic acid derivative 1	-	-	-	-	2.181	-	-	-	0.197	-	-
chlorogenic acid derivative 2	0.150	-	-	-	0.132	-	-	-	-	-	-
chlorogenic acid derivative 3	-	-	-	0.215	-	-	-	-	-	-	-
chrysoeriol derivative 1	-	-	-	0.057	-	-	-	-	-	-	-
chrysoeriol derivative 2	-	-	-	-	-	-	-	-	-	-	0.048
chrysoeriol glycoside	-	-	-	-	-	-	3.455	-	-	3.884	-
cinnamic acid derivative 1	0.134	-	-	-	-	-	-	-	-	-	-
cinnamic acid derivative 2	0.019	-	-	-	0.104	-	-	-	-	-	-
cinnamic acid derivative 3	-	-	-	-	-	-	-	-	-	-	1.094
dicoumaroyl flavonol	-	0.010	0.016	0.104	-	-	-	-	-	-	-
dihydromyricetin	-	-	-	-	-	-	-	-	1.370	-	-
dihydroquercetin	-	-	-	-	-	2.390	-	-	-	-	-
dihydrokaempferol	-	-	-	-	-	-	0.140	-	-	-	-
eriodictyol 7-glucoside	-	-	-	-	-	-	2.444	-	-	5.790	-
eriodictyol aglycon derivative 1	-	-	-	-	-	-	-	-	-	1.219	-
eriodictyol aglycon derivative 2	-	-	-	-	-	-	-	-	-	1.640	-

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
eriodictyol derivative 1	-	-	-	-	-	-	-	-	-	0.752	-
eriodictyol derivative 2	-	-	-	-	-	-	-	-	-	0.249	-
eriodictyol diglycoside 1	-	-	-	-	-	-	2.086	-	-	1.504	-
eriodictyol diglycoside 2	-	-	-	-	-	-	0.601	-	-	1.524	-
eriodictyol diglycoside 3	-	-	-	-	-	-	-	-	-	2.361	-
eriodictyol glycoside	-	-	-	-	-	-	-	-	-	0.483	-
flavonoid diglucoside	-	-	-	-	-	-	-	-	-	-	0.405
hyperin	-	-	-	-	-	3.029	-	-	-	-	1.391
isorhamnetin aglycon derivative 1	0.104	-	-	-	-	-	-	-	-	-	-
isorhamnetin aglycon derivative 2	0.703	-	-	-	-	-	-	-	-	-	-
isorhamnetin derivative 1	-	-	-	-	0.212	-	-	-	-	-	-
isorhamnetin derivative 2	-	-	-	-	-	-	-	-	0.784	0.806	-
isorhamnetin derivative 3	-	-	-	-	-	-	-	-	0.909	-	0.162
isorhamnetin derivative 4	0.473	-	-	-	-	-	-	-	-	-	-
isorhamnetin glycoside 1	0.866	-	-	-	1.548	-	-	-	-	-	-
isorhamnetin glycoside 2	-	-	-	-	1.001	-	-	-	-	-	-
isorhamnetin rhamnoside	2.152	-	-	-	-	-	-	-	0.633	-	-
kaempferol 3-arabinoside	-	-	-	-	1.126	-	-	-	0.064	-	-
kaempferol 3-glucoside	-	-	-	-	0.064	0.395	-	-	0.109	-	2.763
kaempferol 3-rhamnoside	-	-	-	-	-	-	-	-	-	0.401	-
kaempferol glycoside derivative 1	-	-	-	-	1.508	-	-	-	-	-	-
kaempferol glycoside derivative 2	-	-	-	-	-	-	-	-	-	-	0.231
kaempferol glycoside derivative 3	-	-	-	-	-	0.111	-	-	-	-	-
luteolin 5-glucoside	-	-	-	-	0.905	-	-	7.116	-	-	-
luteolin 7-glucoside	-	1.275	0.307	0.313	0.117	-	5.559	5.563	-	1.018	-
luteolin aglycon derivative 1	-	-	-	-	-	-	-	-	-	0.353	-
luteolin aglycon derivative 2	-	-	-	-	-	-	0.355	-	-	1.072	-
luteolin aglycon derivative 3	-	0.022	0.007	-	-	-	-	-	-	-	-
luteolin glycoside 1	-	-	-	-	0.034	-	1.743	-	-	0.718	-

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
luteolin glycoside 2	-	-	-	-	0.080	-	-	-	-	-	-
luteolin glycoside 3	-	-	-	-	0.048	-	-	-	-	-	-
luteolin glycoside 4	-	0.205	0.115	-	-	-	-	-	-	-	-
luteolin glycoside 5	-	0.356	0.237	-	-	-	-	-	-	-	-
luteolin glycoside 6	-	3.951	1.950	-	-	-	-	-	-	-	-
luteolin glycoside 7	-	6.964	3.864	-	-	-	-	-	-	-	-
luteolin glycoside 8	-	0.354	-	-	-	-	-	-	-	-	-
luteolin glycoside 9	-	-	-	-	-	-	-	2.150	-	-	-
methyl-apigenin derivative 1	-	-	-	-	-	-	-	-	-	-	0.107
methyl-apigenin derivative 2	-	-	-	-	-	-	-	-	-	-	0.434
methyl-apigenin derivative 3	-	-	-	-	-	-	-	-	-	-	0.024
methyl-apigenin derivative 4	-	-	-	-	-	-	-	-	-	-	0.054
methyl-luteolin 5-glucoside	-	1.372	0.489	2.380	-	-	-	0.325	-	-	-
methyl-luteolin aglycon	-	0.064	0.323	-	-	-	-	-	-	-	-
methyl-luteolin glycoside 1	-	-	-	-	0.083	-	0.601	-	-	-	-
methyl-luteolin glycoside 2	-	0.130	-	0.073	0.080	-	-	-	-	0.229	-
methyl-luteolin glycoside 3	-	-	0.071	0.006	-	-	0.740	1.272	-	0.158	-
methyl-luteolin glycoside 4	-	-	-	-	-	-	0.447	-	-	-	-
monocoumaroyl astragalin	-	0.944	1.160	4.092	-	-	-	0.159	1.899	-	-
monocoumaroyl flavonol	-	-	0.016	-	-	-	-	-	-	-	-
myricetin 3-arabinoside	-	-	-	-	-	-	-	-	0.051	-	-
myricetin 3-galactoside	-	-	-	0.197	-	0.621	-	-	0.645	-	-
myricetin 3-glucoside	-	-	0.011	0.007	-	2.348	-	-	-	-	0.340
myricetin glycoside	-	-	-	-	-	-	-	-	0.075	-	-
myricitrin	-	-	-	-	-	0.457	-	-	-	-	-
naringenin 7-glucoside	-	-	-	-	-	-	0.501	-	-	2.058	-
neochlorogenic acid	9.406	0.119	0.373	0.515	3.067	14.218	-	0.748	0.266	-	5.349
p-OH-cinnamic acid derivative 1	0.451	0.221	0.187	0.304	0.431	1.540	-	0.263	0.358	-	0.769
p-OH-cinnamic acid derivative 2	0.425	-	0.266	-	0.347	-	-	0.299	0.056	-	0.387

	S. alb x fra	S. aur	S. cap	S. cin	S. fra	S. pen	S. pur	S. ros	S. vim	S. vim x pur	P. tre
p-OH-cinnamic acid derivative 3	-	0.004	-	-	-	-	-	-	-	-	0.015
p-OH-cinnamic acid derivative 4	-	-	0.006	-	-	-	-	-	-	-	-
p-OH-cinnamic acid derivative 5	-	-	0.105	-	-	-	-	-	-	-	-
p-OH-cinnamic acid derivative 7	-	-	-	-	0.035	-	-	-	-	-	-
p-OH-cinnamic acid derivative 8	-	-	-	-	-	-	-	-	-	-	0.017
p-OH-cinnamic acid derivative 9	-	-	-	-	-	-	-	0.122	-	-	-
p-OH-cinnamic acid glucoside	0.267	0.323	-	0.045	0.328	0.681	-	-	-	-	-
protocatechuic acid	-	0.036	0.037	0.134	-	-	-	-	0.078	-	-
quercetin 3-glucoside	0.820	2.311	0.406	1.067	2.558	7.096	0.615	-	2.272	1.543	11.884
quercetin 3-arabinopyranoside	1.321	-	-	0.013	-	2.637	-	-	0.425	-	0.594
quercetin 3-arabinofuranoside	-	-	-	-	-	1.030	-	-	-	-	-
quercetin aglycon	0.584	-	-	-	-	-	-	-	-	-	-
quercetin derivative 1	0.160	-	-	-	-	-	-	-	-	-	-
quercetin diglycoside 1	-	0.393	-	-	-	-	-	-	-	-	2.194
quercetin diglycoside 2	-	-	-	-	0.905	-	-	-	0.099	-	0.998
quercetin diglycoside 3	-	-	-	-	-	-	-	-	0.136	-	-
quercetin diglycoside 4	0.394	0.066	-	-	0.493	-	-	-	0.067	-	-
quercetin glycoside 1	-	-	0.051	-	-	-	-	-	-	-	-
quercetin glycoside 2	-	-	-	-	-	-	-	-	0.055	-	-
quercetin glycoside 3	-	-	-	-	-	-	-	-	0.099	-	-
quercetin triglycoside 1	-	-	-	-	-	-	-	-	0.108	-	-
quercetin triglycoside 2	-	-	-	-	-	-	-	-	0.050	-	0.352
quercetin triglycoside 3	-	-	-	-	-	-	-	-	-	-	0.312
quercitrin	0.765	-	-	-	0.222	0.707	-	-	1.609	2.296	-
rhamnetin aglycon derivative	-	-	-	-	-	-	-	-	-	1.501	-
salipurposide	-	-	-	-	-	-	0.354	-	-	-	-
Condensed tannins	127.260	196.726	139.765	160.468	51.911	192.185	42.617	134.299	138.526	112.270	37.895

Table S2**.** Seasonal variability in secondary metabolite content. The samples for analysis were obtained in early June ("spring") and early August ("summer")

Host-plant species	Salicylates spring (mg/g)	Salicylates summer (mg/g)	Flavonoids spring (mg/g)	Flavonoids summer (mg/g)	Tannins spring (mg/g)	Tannins summer (mg/g)	Salicylates spring (Simpson)	Salicylates summer (Simpson)
Salix (Vetrix) aurita	0.0	0.0	29.5±1.1	21.4±2.3	196.7±45.0	212.5±51.9	0	0
S. (Vetrix) caprea	0.0	0.0	10.6±1.0	6.7±1.4	139.8±37.7	146.2±37.0	0	0
S. (Vetrix) cinerea	0.0	0.0	15.0±2.5	12.6±4.1	160.5±62.2	202.1±48.3	0	0
S. (Salix) fragilis	27.8±9.4	13.8±7.8	25.5±6.5	15.7±7.8	51.9±44.1	55.3±38.0	0.79±0.09	0.45±0.12
S. (Salix) pentandra	41.8±21.5	14.9±10.4	60.6±6.3	49.4±23.8	192.2±34.7	217.9±25.8	0.65±0.2	0.58±0.11
S. (Vetrix) purpurea	164.8±19.1	106.6±45.2	21.3±1.4	24.0±5.9	42.6±59.2	71.3±60.5	0.67±0.06	0.64±0.09
S. (Vetrix) rosmarinifolia	169.0±42.0	132.9±11.0	20.9±0.4	20.4±3.9	134.3±82.9	149.1±46.5	0.64±0.07	0.62±0.03
S. (Vetrix) viminalis	0.0	0.0	16.0±3.4	11.9±4.3	138.5±35.9	121.2±40.3	0	0
S. alba x fragilis	3.4±1.9	0.7±0.9	27.3±4	21.0±6.3	127.3±37.1	133.2±52.0	0.49±0.08	0.24±0.27
S. viminalis x purpurea	2.6±0.7	0.7±0.3	30.5±3.8	35.8±5.0	112.3±24.2	137.0±30.8	0	0
Populus tremula	19.4±20.7	6.0±5.8	33.8±5.9	20.4±5.6	37.9±35.0	57.6±32.1	0.34±0.32	0.38±0.25

Table S3. Correlation between defensive traits of studied willow species. The lower triangle shows values obtained from analysis using phylogenetic generalized least squares, the upper triangle obtained from simple linear regression. Significant values are in bold.

	Salicylate content (mg/g)	**Salicylate Diversity (Simpson)**	**Flavonoid content (mg/g)**	**Tannin content (mg/g)**	**Specific leaf area (cm^2/g)**	**Trichome cover (%)**
	$F_{(6)}$ / p	$F_{(6)}$ / p	$F_{(6)}$ / p	$F_{(6)}$ / p	$F_{(6)}$ / p	$F_{(6)}$ / p
Salicylate content (mg/g)	----------	1.91 / 0.301	1.22 / 0.319	1.35 / 0.298	0.01 / 0.951	**7.37 / 0.042**
Salicylate diversity (Simpson)	1.71 / 0.369	----------	2.57 / 0.160	3.35/ 0.117	3.37 / 0.116	**20.29 / 0.004**
Flavonoid content (mg/g)	1.79 / 0.245	0.26 / 0.777	----------	0.35 / 0.579	0.86 / 0.396	3.00 / 0.143
Tannin content (mg/g)	2.84 / 0.136	2.97 / 0.127	0.21 / 0.816	----------	0.15 / 0.718	2.08 / 0.215
Specific leaf area (cm^2/g)	>0.01 / 0.999	3.30 / 0.108	2.09 / 0.205	0.13 / 0.880	----------	0.19 / 0.678
Trichome density (%)	**7.98 / 0.020**	**18.36 / 0.003**	4.19 / 0.073	4.34 / 0.066	0.47 / 0.647	----------

Table S4. Impact of *Salix* defensive traits on leaf-chewing insect diversity and density. Table includes results of simple linear regression and phylogenetic generalized least squares (PGLS). Significant values are in bold.

	Diversity		**Density**	
	$F_{(9)}$ / p	PGLS $F_{(9)}$ / p	$F_{(9)}$ / p	PGLS $F_{(9)}$ / p
Salicylate content (mg/g)	**5.52 / 0.043**	**4.98 / 0.035**	0.03 / 0.866	0.06 / 0.945
Salicylate diversity (Simpson)	2.46 / 0.152	1.74 / 0.230	0.14 / 0.719	0.14 / 0.719
Flavonoid content (mg/g)	3.34 / 0.101	2.83 / 0.111	0.20 / 0.668	0.35 / 0.712
Tannin content (mg/g)	0.17 / 0.687	0.05 / 0.951	0.12 / 0.738	0.50 / 0.621
Specific leaf area (cm^2/g)	0.42 / 0.683	0.60 / 0.570	1.55 / 0.244	1.45 / 0.286
Trichome density (%)	3.47 / 0.095	2.68 / 0.123	1.03 / 0.336	1.53 / 0.268