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**Specialised chemistry affects insect abundance but not overall
community similarity in three rare shrub willows: *Salix
myrtilloides*, *S. repens* and *S. rosmarinifolia***

Rigorózní práce

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Annotation

This study investigates the relationship between the unique chemical profiles of three willow species (*Salix myrtilloides*, *S. repens*, and *S. rosmarinifolia*) and the diversity of their insect herbivore communities. These willows, which grow in ecologically significant areas within the Czech Republic, are examined to understand how their specialized metabolites influence associated insect populations. Utilizing non-targeted metabolomics, we analysed the overall metabolomic profiles and specifically the presence of salicinoids within these plants. Additionally, we conducted surveys of various insect groups including caterpillars, leaf-chewing beetles (both adults and larvae), sawfly larvae, and sap-sucking Hemiptera. Through non-targeted metabolomics, we identified over 2,000 metabolites, highlighting a unique chemical profile for *S. myrtilloides* and higher salicinoid levels in *S. repens* and *S. rosmarinifolia*. Abundance of generalist herbivores was greater on *S. myrtilloides*, whereas specialist herbivores were evenly distributed among all willow species. The study found that insect community composition was similar among the willow species, suggesting that salicinoids might deter generalist herbivores but do not significantly impact overall insect diversity.

Declaration:

I declare that I am the author of this qualification thesis and that I have used only the sources and literature indicated in the list of sources for the preparation of this thesis.

České Budějovice 08.3. 2024

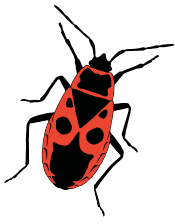
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I declare that I participated significantly in the publication by data curation, statistical analysis and original draft writing.

My total share in this publication was 50 %.



Specialised chemistry affects insect abundance but not overall community similarity in three rare shrub willows: *Salix myrtilloides*, *S. repens* and *S. rosmarinifolia*

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Key words. Insects, herbivore, host plants, concentration and richness of metabolites, salicinoids, Salicaceae, *Salix*, secondary metabolites, Czech Republic

Abstract. Willows serve as a keystone host-plant genus for insect herbivores. The diversity of insect herbivore assemblages harboured by willows is typically affected by the diversity of specialised metabolites that willows produce. Here, we studied three small, shrubby willow species (*Salix myrtilloides*, *S. repens* and *S. rosmarinifolia*) that primarily occur at sites of high conservation value in the Czech Republic. We explored if associated insect communities reflect the specialised chemistry in these uncommon host plants. We measured the three willow species for overall metabolomic profiles and salicinoids using non-targeted metabolomics and sampled them for caterpillars, leaf-chewing beetles (adults and larvae), sawfly larvae, and sap-sucking Hemiptera. We detected 2,067 metabolites across the three willow species. Most of them were shared by *S. repens* and *S. rosmarinifolia*, while *S. myrtilloides* showed a distinct chemical profile. *Salix repens* and *S. rosmarinifolia* also had significantly higher concentration and richness of salicinoids than *S. myrtilloides*. The abundance of all insect species and generalists that also feed on host-plants outside Salicaceae was higher on *S. myrtilloides* than on *S. rosmarinifolia* or *S. repens*. The abundance of Salicaceae specialists did not differ among the three willow species. Insect community composition, in contrast, did not show pronounced differences among the three willows. Our results suggest that salicinoids may be responsible for the low abundance of generalist herbivores. Furthermore, our study indicates that herbivore community composition does not reflect the specialised chemistry in the three willows we studied. Therefore, we hypothesise that the presence of some of the insect species is primarily determined by other factors, such as the habitat type where the respective willow species occur. Although the studied willows possess some characteristic specialised chemistry, we conclude that their importance as hosts of specific and sometimes threatened insect fauna may be mediated by willow habitat preference.

INTRODUCTION

The plant genus *Salix* Linnaeus, 1753 (willows, also called sallows and osiers) contains around 450 species worldwide (Skvortsov, 1999; Argus, 2009). They display a wide range of variation in life history, which has allowed them to colonise diverse environments over a broad geographic range. Most species occur in temperate, subarctic, and arctic regions of the Northern Hemisphere, while only

a few species occur in tropical South America, Africa, and Southeast Asia (Skvortsov, 1999; Argus, 2009). In Europe, *Salix* represents one of the most speciose genera of woody plants, being distributed from lowland floodplains to alpine habitats at high elevations (Hörandl et al., 2012). Due to their outstanding diversity and broad distribution, willows serve as a keystone host-plant genus for insect herbivores (Narango et al., 2020).

Willows possess various specialised (or secondary) metabolites that serve as defences against herbivores, pathogens, or detrimental abiotic factors (Tegelberg & Julkunen-Tiitto, 2001; Hjalten et al., 2007). Willow metabolites include mainly various phenolics, such as flavonoids, tannins, or salicinoids (Hjalten et al., 2007; Volf et al., 2015a). Salicinoids, in particular, have been studied for their anti-herbivore roles in willow defence (Volf et al., 2015a). The effect of salicinoids is tightly linked to the specialization of insect herbivores. Generalist herbivores that commonly feed on other hosts than willows are often deterred or otherwise negatively affected by high salicinoid concentration (Denno et al., 1990). In contrast, specialised herbivores have adapted to salicinoids and can use salicinoids as oviposition or feeding cues (Kolehmainen et al., 1995). Some highly adapted herbivore species are even known to sequester salicinoids and use them for their own benefit, a phenomenon which is probably responsible for the loss of salicinoids in some willow species (Volf et al., 2015b). For example, larvae of various *Chrysomela* Linnaeus, 1758, leaf-beetle species can turn salicinoids into salicylaldehyde, which they excrete from their thoracic and abdominal glands in order to repel invertebrate predators (Pasteels et al., 1983).

Variation in the responses of insect herbivores to salicinoids and other specialised metabolites found in willows causes a substantial turnover in herbivore assemblages among willow species with different specialised chemistry (Volf et al., 2015a). Although rigorous studies on the evolution of willow defences are still missing, variable levels of evolutionary conservatism in the chemical composition across the willow phylogeny have been suggested (Julkunen-Tiitto, 1989; Volf et al., 2015b). In other words, closely related species tend to be similar in their chemistry, while distantly related taxa show pronounced differences in their specialised metabolites – a pattern that has been previously recovered in several speciose plant genera (e.g. Endara et al., 2017; Volf et al., 2018). Furthermore, the highly characteristic chemistry of several willow species can probably explain why this plant genus harbours a highly diverse insect fauna of which many species are strictly monophagous (Nyman et al., 2006; Volf et al., 2015a). Understanding variation in willow specialised chemistry and its evolution can thus unveil important factors that made willows keystone hosts for insect herbivores (Volf et al., 2015a; Narango et al., 2020).

In addition to their diverse chemistry, willows also show a high variability in their growth forms (Skvortsov, 1999; Argus, 2009; Hörandl et al., 2012), including trees, shrubs, and dwarf shrubs. In Central European lowland, most species grow as trees or large shrubs, whereas dwarf shrubs become more prevalent at higher elevations (Skvortsov, 1999; Hörandl et al., 2012). However, a few species grow as small shrubs (<1–1.5 m tall) in Central European lowlands. These species usually exhibit limited geographic distributions due to their associations with threatened, spatially highly isolated habitats, such as bogs and wet meadows (Hörandl et al., 2012; Vašut et al., 2013). These wil-

low species are known to host various specialised insect herbivores seldom found on other host-plants or in other habitats (Macek et al., 2007, 2020). In this exploratory study, we focused on three such willow species, i.e., *Salix myrtilloides* L., *Salix repens* L., and *Salix rosmarinifolia* L. All three species are small shrubs that typically occur in mesotrophic wetlands, edges of *Sphagnum* L. bogs or damp meadows from lowlands to highlands (Skvortsov, 1999).

Salix myrtilloides is widely distributed in Northern Eurasia with its continuous distribution reaching from the Far East to Scandinavia, the Baltic States, and eastern Poland. Its distribution in Central Europe is highly fragmented. In the Czech Republic, this species is critically endangered and currently known only from four sites, including the relatively recently discovered sites in the Šumava National Park (Vašut et al., 2013; Lepší & Lepší, 2014; Grulich & Chobot, 2017). It belongs to a different section of *Salix* than the other two species and it seems to differ also in its specialised chemistry (Julkunen-Tiitto, 1989; Skvortsov, 1999). *Salix repens* and *S. rosmarinifolia* are closely related, sometimes being considered two subspecies of a single species. Previous studies have shown that they share largely similar salicinoid profiles, although studies on their overall chemical similarity are not available (Julkunen-Tiitto, 1989). *Salix repens* occurs mainly in Western and Northern Europe, while the distribution of *S. rosmarinifolia* stretches from Central Europe eastwards and reaches Mongolia and northern China (Skvortsov, 1999). The two (sub-)species form a hybrid zone in the Czech Republic, with *S. repens* occurring primarily in Western and Northern Bohemia and locally in the Bohemian-Moravian Highlands. The distribution of *S. rosmarinifolia* is broader and includes also South Bohemia and eastern parts of the country (Vašut et al., 2013). Both *S. repens* and *S. rosmarinifolia* are considered to be vulnerable in the Czech Republic (Grulich & Chobot, 2017).

For the studied willows, we characterised the overall metabolomic profiles and measured the salicinoid concentration using untargeted metabolomics. We further studied their insect herbivore communities, including caterpillars, leaf-chewing beetles (adults and larvae), sawfly larvae, and sap-sucking Hemiptera. We predicted that (i) *S. repens* and *S. rosmarinifolia* would show largely similar specialised chemistry, (ii) willows with high salicinoid concentration would harbour less abundant herbivore communities, and (iii) the similarity of herbivore communities associated with the three willow species would reflect their chemical similarity, and should thus be higher between *S. repens* and *S. rosmarinifolia*. Taken together, our study explores the insect communities and specialised chemistry in these uncommon host-plants and discusses to what extent they might be related.

MATERIALS AND METHODS

Study sites and field sampling

Our study was conducted at thirteen sites in South and West Bohemia and one site in the Bohemian-Moravian Highlands (at

altitudes of 508–774 m a.s.l.), situated in mesophytic and oreophytic phytogeographical regions. The sites were selected according to the occurrence and abundance of the focal willow species. The sites are characterised as lowland wetlands, wet meadows and forest edges (Table S1). Six of the sampling sites are protected as nature reserves, illustrating that the focal willow species frequently inhabit sites of a high conservation value.

Within each site, we surveyed the local willow population and sampled all available plant individuals for insect herbivores. In total, our dataset included eight individuals (or polycormones) of *S. myrtilloides*, 24 individuals of *S. repens* and 52 individuals of *S. rosmarinifolia*. The sampling was conducted in 2020 (April–June) and 2021 (June) at all studied localities (Table S1). Data from both years were pooled. We sampled the main leaf-feeding insect groups, including caterpillars (Lepidoptera), leaf-chewing beetles and larvae (Coleoptera), sawfly larvae and galls (Hymenoptera), as well as sap-sucking Hemiptera. Identified Hemiptera included aphids (Aphidoidea), jumping plant lice (Psylloidea), and true hoppers (Auchenorrhyncha). To obtain the insects, we sampled the foliage using a 45 cm-diameter sweeping net. The sweeping method was supplemented by a direct manual search as described in Volf et al. (2015a). We recorded the sampling effort of sweeping and manual searching to later standardise insect abundance. To do so, we estimated the number of leaves sampled at each site per host and photographed 50 leaves for each of the three willow species to calculate an average area of a single leaf. We calculated the leaf area sampled for insects by extrapolating the average area of one leaf by the number of leaves sampled for insects.

When we encountered extremely high abundance of a single insect species (i.e. several hundred conspecific individuals sampled from a single plant), we collected ca. 20% of the individuals and extrapolated the count numbers to estimate the overall abundance. Leaf-chewing beetles and sap-sucking Hemiptera were stored in 96% ethanol and identified to species level using available literature. Caterpillars and sawfly larvae were morphotyped, photographed for further identification to species level and stored in 96% ethanol. Some larvae were reared to adults for further identification. Additionally, 35 individuals were barcoded at the Canadian Centre for DNA Barcoding (CCDB, Guelph, Canada). COI sequences (658 bp) were uploaded to the Barcode of Life Database (BOLD, www.boldsystems.org). Species identifications were validated based on the assigned Barcode Index Numbers (Ratnasingham & Hebert, 2013).

Using available literature (Ossiannilsson, 1992; Nickel, 2003; Čížek, 2006; Zahradník, 2008; Macek et al., 2007, 2008, 2012, 2015, 2020; Liston et al., 2017; Laštůvka et al., 2018), we categorised the sampled taxa as Salicaceae specialists (species feed-

ing only on host plants in the Salicaceae family) or generalists (species feeding not only on Salicaceae but also on other plant lineages). Species which were not previously reported to feed on Salicaceae based on available literature (i.e., transient taxa without trophic association) (Ossiannilsson, 1992; Nickel, 2003; Čížek, 2006; Zahradník, 2008; Macek et al., 2007, 2008, 2012, 2015, 2020; Liston et al., 2017; Laštůvka et al., 2018) were excluded from further statistical analyses, since their feeding habits do not reflect willow biology.

Furthermore, we sampled a subset of leaves to analyse the specialised chemistry of each willow species. To do so, we sampled 20 healthy, undamaged, freshly matured leaves from four individuals of *S. myrtilloides*, five individuals of *S. repens*, and five individuals of *S. rosmarinifolia* (Table 1). The leaves were stored in liquid nitrogen, freeze-dried and homogenised. The homogenised tissue was used in the subsequent chemical analyses. We avoided sampling leaves from obviously clonal individuals, to capture intraspecific variation and to avoid pseudo-replication in the chemistry.

Leaf chemistry

We performed untargeted metabolomics to quantify specialised chemistry of the studied willows. Specialised metabolites were extracted from ca 10 mg (measured to within 0.01 mg accuracy) of homogenised material using 1.8 ml 90:10 (v/v) methanol/water solvent overnight at 4°C while shaking at 300 rpm. After that, they were centrifuged at 14,000 rpm for 30 min, and the supernatant removed and filtered for analysis using liquid chromatography-mass spectrometry (LC-MS) (Sedio et al., 2021). We optimised UHPLC-MS parameters to detect and fragment metabolites representing a wide range in polarity and mass (Sedio et al., 2018). Metabolomic extracts were separated using a Thermo Fisher Scientific (Waltham, MA, United States) Vanquish Horizon Duo ultra-high performance liquid chromatography (UHPLC) system with an Accucore C18 column with 150 mm length, 2.1 mm internal diameter, and 2.6 µm particle size. UHPLC buffer A (0.1% v/v formic acid in water) and buffer B (0.1% v/v formic acid in methanol) were employed in a solvent gradient from 5 to 100% buffer B over 18 min. Separation of metabolites by UHPLC was followed by heated electrospray ionization (HESI) in positive mode using full scan MS1 and data-dependent acquisition of MS2 (dd-MS2) on a Thermo Fisher Scientific Q Exactive hybrid quadrupole-orbitrap mass spectrometer. We analysed two types of samples: extracts from individual trees and species pools, consisting of pooled aliquots of all conspecific individuals for each species. For individuals, we collected a MS1 scan (115–1,725 m/z) at a resolution of 140,000. For species pools, the MS1 full scan was at 70,000 resolution, followed by dd-MS2 at 17,500

Table 1. Willow individuals analysed for specialised chemistry.

Species	Individual	Site	GPS coordinates
<i>S. myrtilloides</i>	MYR1	West Bohemia, Prameny, NPP Upolínová louka pod Křížky	N50°4.0913' E12°44.746'
<i>S. myrtilloides</i>	MYR2	South Bohemia, Chlum, Luh u Volar	N48°51.610' E13°53.924'
<i>S. myrtilloides</i>	MYR3	South Bohemia, Chlum, Luh u Volar	N48°51.686' E13°53.926'
<i>S. myrtilloides</i>	MYR4	South Bohemia, Chlum, Luh u Volar	N48°51.679' E13°54.045'
<i>S. repens</i>	REP2	West Bohemia, Lesná, NPP Na požárech	N49°42.074' E12°28.144'
<i>S. repens</i>	REP3	West Bohemia, Lesná, NPP Na požárech	N49°42.111' E12°27.568'
<i>S. repens</i>	REP4	West Bohemia, Stará Knížecí Huť	N49°42.830' E12°26.485'
<i>S. repens</i>	REP5	West Bohemia, Stará Knížecí Huť	N49°42.829' E12°26.493'
<i>S. repens</i>	REPx1	Bohemian-Moravian Highlands, Háj u Horních Rápotic	N49°34.948' E15°18.399'
<i>S. rosmarinifolia</i>	ROS1	South Bohemia, Ohrazení, NP Ohrazení	N48°56.923' E14°37.751'
<i>S. rosmarinifolia</i>	ROS2	South Bohemia, Milíkovice, NP Děkanec	N48°51.978' E14°26.451'
<i>S. rosmarinifolia</i>	ROS3	South Bohemia, Milíkovice, NP Děkanec	N48°51.974' E14°26.459'
<i>S. rosmarinifolia</i>	ROS4	South Bohemia, Milíkovice, NP Děkanec	N48°52.004' E14°26.433'
<i>S. rosmarinifolia</i>	ROS5	South Bohemia, Milíkovice, NP Děkanec	N48°51.998' E14°26.417'

resolution on the five most abundant precursors found in the MS1 spectrum. Automatic gain control target values were $1e6$ for full scan MS1 and $1e5$ for dd-MS2. Maximum ion injection times were 200 ms for full scan MS1, 100 ms for QC MS1, and 50 ms for MS2. For dd-MS2, we set the isolation window to 1.5 m/z and stepped collision energy at 20, 40, and 60. QC pooled samples were used to account for fluctuations in total ion intensity due to changes in temperature and atmospheric pressure over time.

Raw data from the UHPLC-MS analyses were centroided and processed for peak detection, peak alignment, and peak filtering using MZmine2 (Pluskal et al., 2010), which groups chromatographic features into putative compounds based on molecular mass and LC retention time. We used the same parameters as Sedio et al. (2021) except for setting MS1 noise threshold to 15,000 ion counts and MS2 noise threshold to 1,500 ion counts. In addition to quantifying overall differences based on all detected metabolites, we were particularly interested in differences in salicinoids. We inferred molecular formulae using Sirius (Dührkop et al., 2019), predicted structures using CSI:fingerID (Dührkop et al., 2015) and classified the metabolites using CANOPUS (Dührkop et al., 2021). Various salicinoids were classified by CANOPUS as belonging to different metabolite classes, namely to alkyl glycosides, coumaric acid esters, O-glycosyl compounds, phenol ethers, and phenolic glycosides. As these groups include numerous other metabolites unrelated to salicinoids, we used the structures predicted by CSI: FingerID and SIRIUS, represented as SMILES, to manually subsample these five metabolite groups for salicinoids plus their derivatives that we used in the subsequent analyses. We used the network-visualization software Cytoscape (Shannon et al., 2003) to illustrate structural similarities among individual metabolites in the molecular network and to visualize how they are shared among the studied willows. Compounds such as contaminants and industrial surfactants that occurred in blanks (i.e. in control samples that did not contain any leaf tissue) were removed from further analyses. To obtain the blank samples, we carried out the extraction process using the same mate-

rial and solvents as in the case of leaf samples, but without adding any leaf tissue.

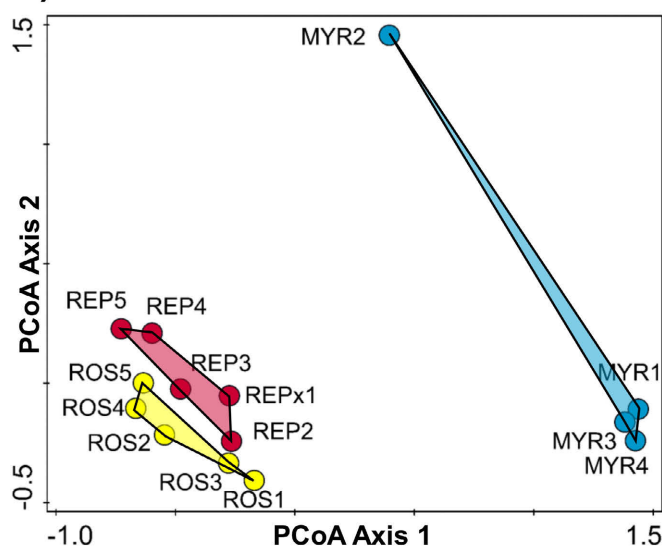
We also used the network to calculate the chemical structural-compositional similarity (CSCS) metric, which provides a chemical similarity score that accounts for the structural similarity of metabolites for every pair of samples in the three datasets following Sedio et al. (2017). We then calculated structural variation between samples as 1-CSCS. The use of CSCS to measure similarity among samples based on untargeted MS/MS data provides a chemically meaningful measure of structural variation in metabolite composition. Consider an example: Compounds x and y are structurally similar, sample A contains x but not y , and sample B contains y but not x . In this example, compounds x and y would contribute zero to indices based solely on presence or concentration of metabolites (e.g. Bray-Curtis dissimilarity) but make a positive contribution to CSCS. We calculated CSCS for all the metabolites detected and for salicinoids plus their derivatives separately.

Statistical analysis

First, we compared specialised chemistry across the three studied willow species. We visualised the metabolite similarity based on CSCS matrices for all metabolites and salicinoids plus their derivatives detected by untargeted metabolomics across the studied samples using PCoA. Ordination analyses were performed in Canoco 5.12 (ter Braak & Šmilauer, 2012). Additionally, we compared the concentration (as the sum of the area under chromatographic peaks/mg) and richness (as the number of metabolites) of salicinoids and their derivatives among the three willow species using ANOVA. We log-transformed the concentration and used Tukey post-hoc tests to test for significant pairwise differences among the three willow species using R 3.6.1 (R Core Team, 2019).

Second, we compared abundance of insect herbivores among the hosts with ANOVA. We used sites as samples (each site harboured only one of the three host species). In particular, we used

A) All metabolites



B) Salicinoids

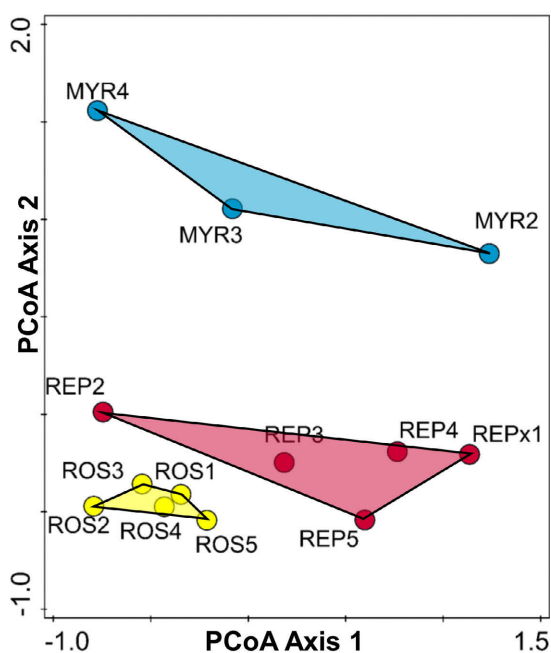


Fig. 1. Chemical similarity with respect to all metabolites (A) and salicinoids plus their derivatives (B) across *Salix myrtilloides*, *S. repens*, and *S. rosmarinifolia* as detected by untargeted metabolomics and visualized by PCoA. Chemical similarity was based on CSCS matrices. The first two axes explained 94.5% and 96.5% of the variation with respect to all metabolites and salicinoids, respectively.

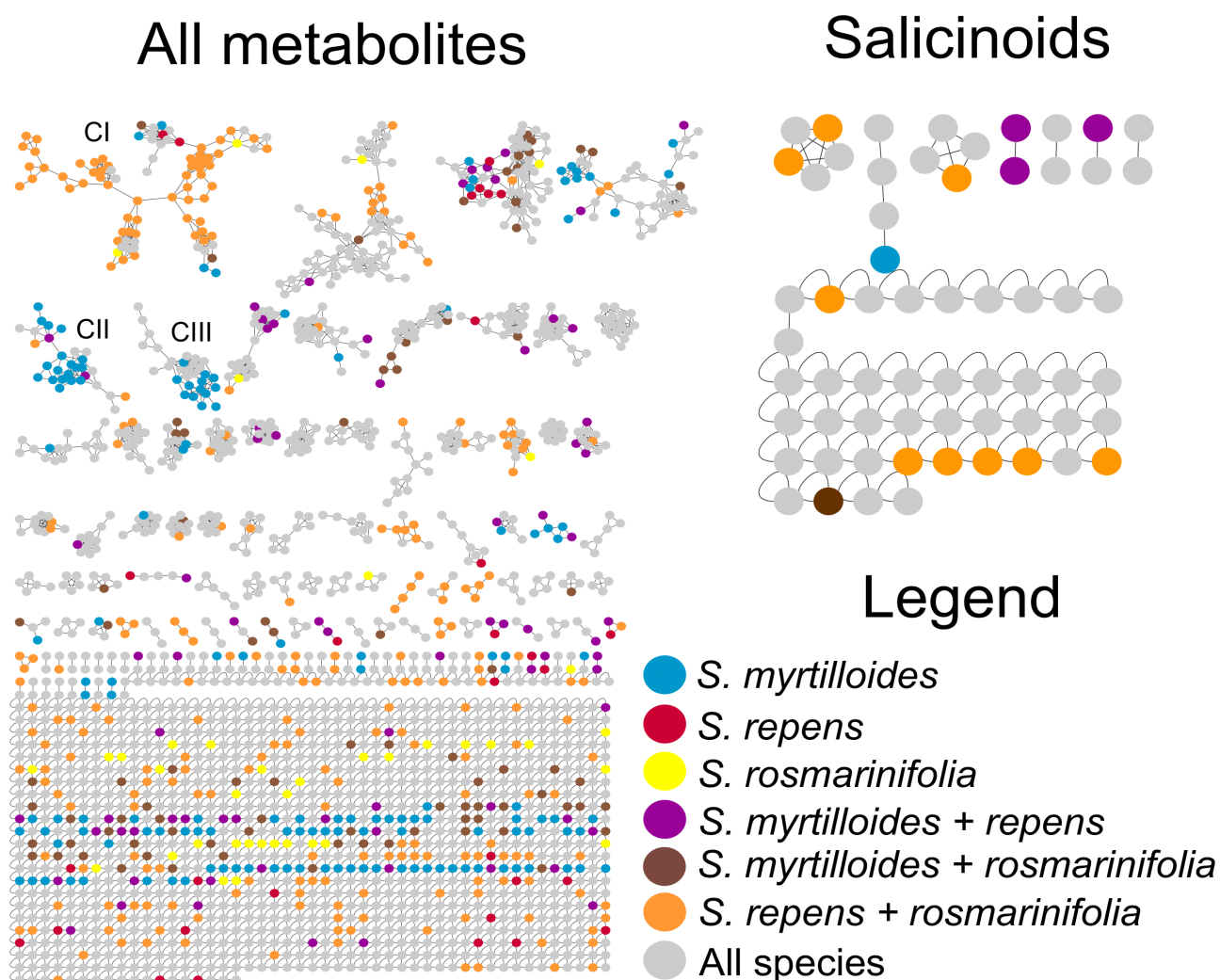


Fig. 2. All metabolites and salicinoids detected in *Salix myrtilloides*, *S. repens*, and *S. rosmarinifolia* by untargeted metabolomics. The metabolites are shown as individual dots. They are joined into clusters based on their MS spectra. Cluster I (CI) represents mainly O-glycosyl compounds and phenolic glycosides occurring exclusively in *S. repens* and *S. rosmarinifolia*. CII represents phenolic glycosides and CIII represents biflavonoids and polyflavonoids occurring exclusively in *S. myrtilloides*. Structurally distinct metabolites that do not link to other metabolites appear as isolated dots at the bottom of diagrams. Colour-coding indicates how the metabolites are shared across the studied willow species.

4 samples for *S. myrtilloides*, 5 samples for *S. repens*, and 5 samples for *S. rosmarinifolia*. We used host identity as the explanatory variable, and insect abundance as the response variable. We standardised insect abundance with the sampled leaf area and log-transformed it. When the overall model revealed significance, we used Tukey post-hoc tests to test for significant pairwise differences among the three willow species. The analyses were performed separately for all insect species, generalists, and specialists using R 3.6.1 (R Core Team, 2019).

Finally, we compared insect community composition among the three willows to explore if the patterns are related to variation in specialised chemistry. We calculated Bray-Curtis dissimilarity distances of standardised abundance on sites and performed PCoA, with elevation as a covariable. The analyses were performed three times separately: (1) across all species, (2) for generalists only, and (3) for specialists only. To further account for the possible effect of geography on the insect community composition, we also tested for the effect of geographic distances between the sites on insect communities. We used GPS coordinates of the individual sites to calculate a dissimilarity matrix based on their spatial distances. We transformed the distance matrix

into PCoA axes (Gower, 1966) and tested their effect on insect community composition (proportion of insect species to a given site) using a CCA with forward selection and 999 permutations. Ordination analyses were performed in Canoco 5.12 (ter Braak & Šmilauer, 2012).

RESULTS

We detected 2,067 metabolites across the three willow species. Out of these, 62 metabolites were classified as salicinoids or their derivatives. PCoA of all metabolites (Fig. 1A) and salicinoids separately (Fig. 1B) showed that chemical profiles of *S. repens* and *S. rosmarinifolia* based on CSCS matrices are more similar to each other than to the largely different chemical profiles of *S. myrtilloides*. The metabolites occurring uniquely in *S. myrtilloides* included mainly biflavonoids, polyflavonoids, and phenolic glycosides other than salicinoids. The metabolites occurring mainly in the *S. repens* and *S. rosmarinifolia* group included mainly O-glycosyl compounds and phenolic glycosides. This trend was also apparent in the metabolomic

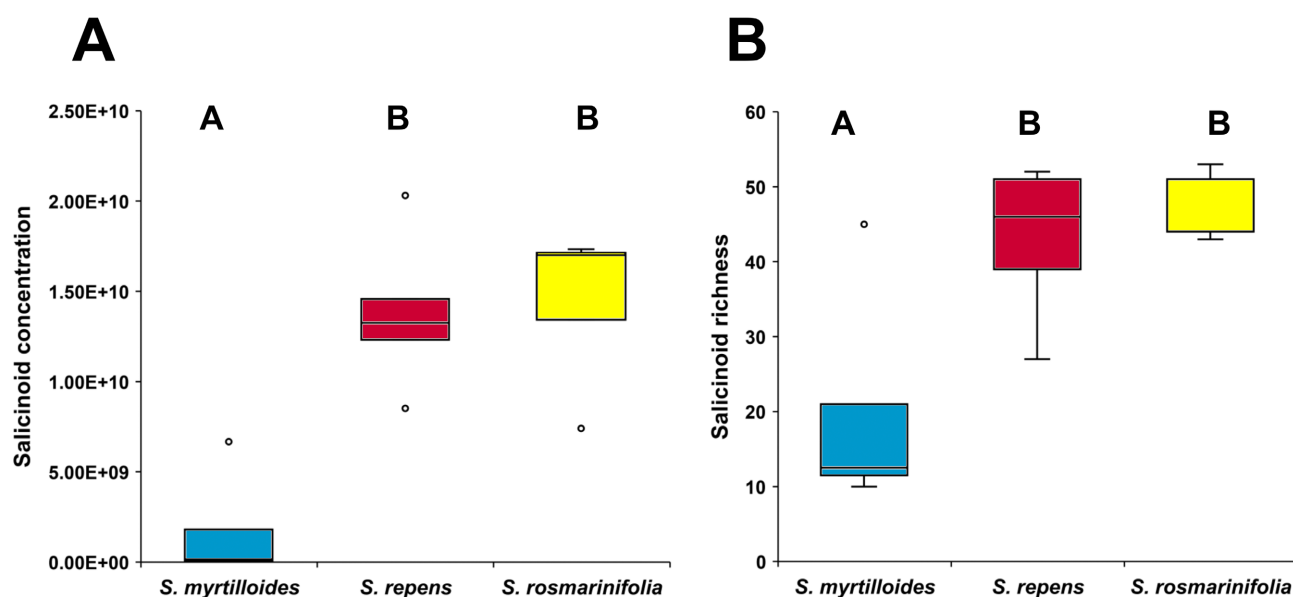


Fig. 3. Differences in the average concentration (the sum of area under chromatographic peaks/mg) (A) and richness (B) of salicinoids and their derivatives among *Salix myrtilloides*, *S. repens*, and *S. rosarinifolia*. The boxes show the first to third quartile with the medians as horizontal lines, the whiskers show range. The concentration of salicinoids and richness significantly differed ($F_{(2,11)} = 14.4$, $p = 0.0008$; $F_{(2,11)} = 9.1$, $p = 0.0046$, respectively). Significant differences between individual willow species indicated by post-hoc Tukey tests are marked with capital letter above the boxes. Willow species are depicted by respective colour (*Salix myrtilloides* blue, *S. repens* red and *S. rosarinifolia* yellow).

networks where the largest cluster (Cluster I) included mainly O-glycosyl compounds and phenolic glycosides occurring exclusively in *S. repens* and *S. rosarinifolia*. Two of the smaller clusters contained phenolic glycosides (Cluster II) and biflavonoids and polyflavonoids (Cluster III) occurring exclusively in *S. myrtilloides* (Fig. 2).

Salix repens and *S. rosarinifolia* contained significantly higher concentrations of salicinoids than *S. myrtilloides* ($F_{(2,11)} = 14.4$, $p = 0.0008$; Fig. 3A). Similarly, salicinoid richness was significantly higher in *S. rosarinifolia* and *S. repens* than in *S. myrtilloides* ($F_{(2,11)} = 9.1$, $p = 0.0046$; Fig. 3B).

In total, we sampled 1,152 insect individuals belonging to 74 species. Out of these, there were 24 species of Lepidoptera, 7 species of Hymenoptera, 28 species of Coleoptera, and 15 species of Hemiptera. The abundance of all insect species and generalists, but not Salicaceae specialists, differed among the willow species ($F_{(2,11)} = 4.3$, $p = 0.0422$ for all species; $F_{(2,11)} = 6.6$, $p = 0.0129$ for generalists; $F_{(2,11)} = 1.3$, $p = 0.3182$ for specialists). When analysed with post-hoc tests, the abundance of all insects was higher on *S. myrtilloides* than on *S. rosarinifolia* (Fig. 4A), the abundance of generalists was higher on *S. myrtilloides* than on both *S. rosarinifolia* and *S. repens* (Fig. 4B), and the abundance of Salicaceae specialists did not differ across the studied willows (Fig. 4C).

The insect communities associated with *S. repens* were highly variable. This variation was retained even when we included altitude as a covariable. The other two willow species showed much smaller variation in insect community composition, possibly due to the small number of sites analysed. In the analysis using all insect species, the insect community composition associated with *S. myrtilloides*

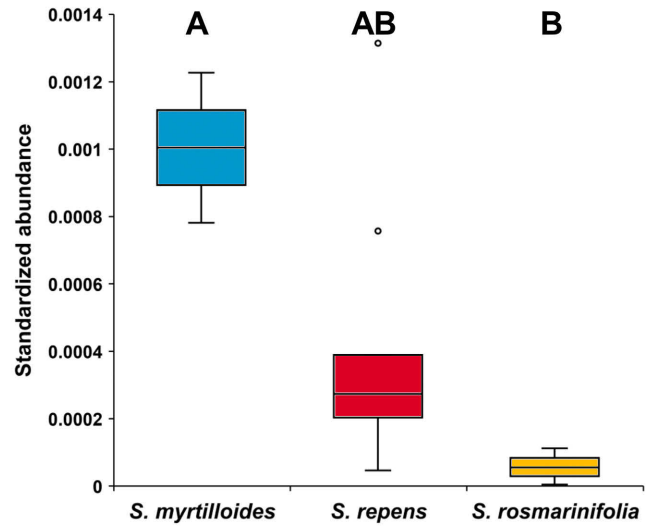
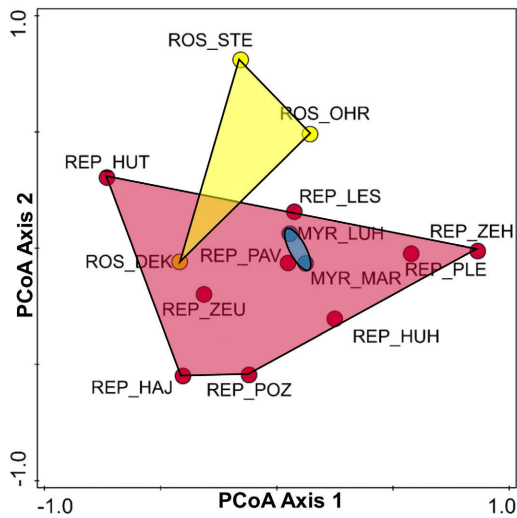
and *S. repens* overlapped, while the community associated with *S. rosarinifolia* formed a partly separated cluster. In the analysis using only generalists, *S. rosarinifolia* was fully separated, whereas it fully overlapped with the two other willow species in the analysis using only specialist insects (Fig. 4). None of the PCoA axes representing spatial distance between the studied sites displayed a significant correlation with insect community composition.

DISCUSSION

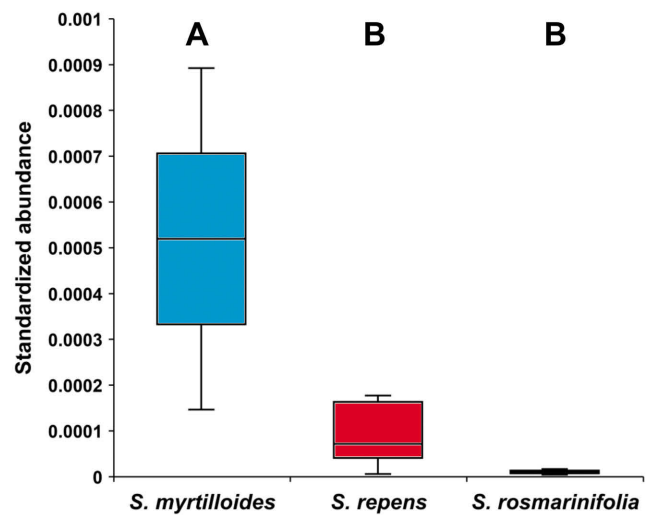
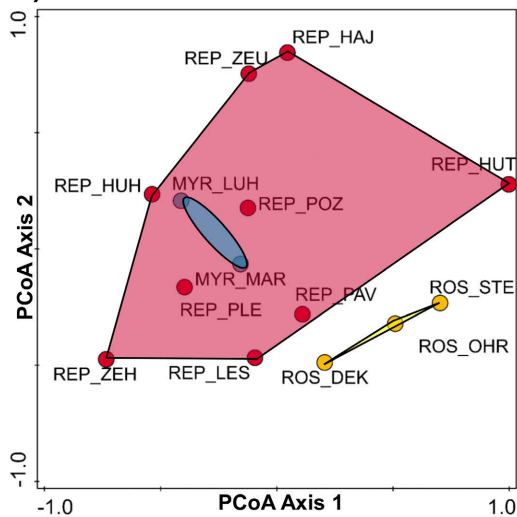
We studied three small shrub willows (*Salix myrtilloides*, *S. repens* and *S. rosarinifolia*) that primarily occur at sites of high conservation value in the Czech Republic (Vašut et al., 2013). We show that *S. repens* and *S. rosarinifolia* share similar chemistry that probably affects the abundance of insect herbivores they harbour as compared to *S. myrtilloides*. In contrast, our results suggest that the overall herbivore community composition did not reflect the host chemistry. Hence, other factors, such as habitat type, might play a more important role in determining herbivore communities of the investigated willow species.

In almost all of the detected metabolite classes, the majority of compounds were shared between *S. repens* and *S. rosarinifolia*. This was particularly apparent in the case of salicinoids, with *S. repens* and *S. rosarinifolia* showing similarly high concentration and diversity of these metabolites. Previous studies suggested that *S. repens* and *S. rosarinifolia* are closely related (He et al., 2021) or, in fact, may be considered a single species (Wagner et al., 2021). Here we show that these two willows also possess largely similar chemical profiles across various metabolite classes. In contrast, we found pronounced differences in specialised chemistry between *S. myrtilloides*, on one

A) All species



B) Generalists



C) Specialists

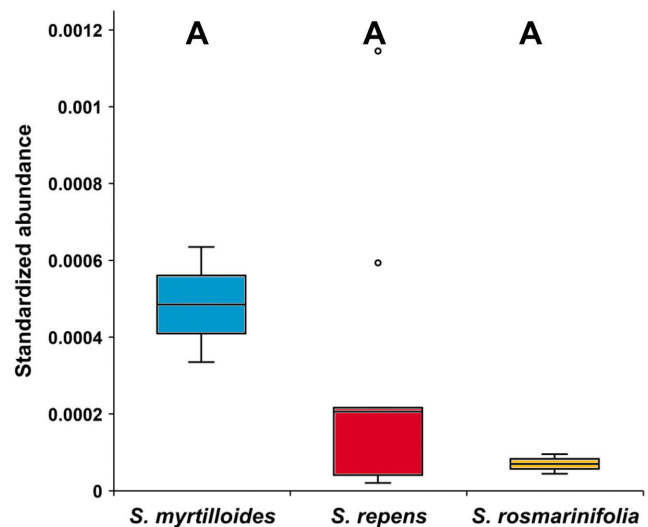
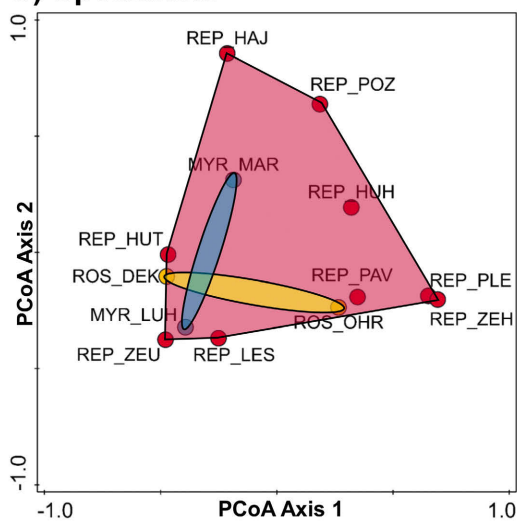


Fig. 4. Insect species composition and abundance among the studied hosts. Left: Ordination plot of the PCoA of the assemblages of all species (A), generalists (B) and specialists (C). Localities are labelled as circles. Willow species are depicted by their respective colour (*Salix myrtilloides* blue, *S. repens* red and *S. rosmarinifolia* yellow). Dissimilarity was calculated based on the Bray-Curtis measure. Right: Differences in abundance of all species (A), generalists (B) and specialists (C) among *Salix myrtilloides*, *S. repens*, and *S. rosmarinifolia* based on linear models. The boxes show the first to third quartile with the medians as horizontal lines, the range indicated by whiskers. Significant differences between individual willow species identified by Tukey post-hoc tests are marked with capital letter above the boxes.

hand, and *S. repens* and *S. rosmarinifolia* on the other. The studied willow species partly occupy similar habitats and show similar growth forms (Skvortsov, 1999; Hörandl et al., 2012). Yet, we found multiple metabolites that were uniquely present in either *S. myrtilloides* or *S. repens* and *S. rosmarinifolia*. These metabolites belonged to several classes, such as various flavonoids that may protect these willows against adverse abiotic conditions in open habitats, and against UV irradiation in particular (Tegelberg & Julkunen-Tiitto, 2001).

The high concentration of salicinoids in *S. repens* and *S. rosmarinifolia* may possibly explain the differences in insect abundance between these two willows and *S. myrtilloides*. Salicinoids can deter generalist insect herbivores from feeding or increase their mortality (Denno et al., 1990). In contrast, specialised herbivores, such as various leaf beetles or sawflies, can sequester them or at least partly avoid their negative effects (Pasteels et al., 1983; Kolehmainen et al., 1994, 1995). As a result, some specialised herbivores prefer feeding and ovipositing on willows with high salicinoid concentration, which may have supported the loss of salicinoids in some derived willow species (Kolehmainen et al., 1995; Volf et al., 2015b). Here we showed that *S. repens* and *S. rosmarinifolia*, with high salicinoid concentration, harboured lower abundance of generalists, than in *S. myrtilloides* while the abundance of Salicaceae specialists did not differ between the three willows.

In contrast to the trends in insect abundance, insect community composition seemingly did not reflect the specialised chemistry in the three willow taxa. In our previous study, we showed that willow specialised chemistry structures communities of leaf-chewing herbivores associated with common species of lowland willows in the region (Volf et al., 2015a, 2018). Here, the species composition of insect herbivores overlapped to a large extent among the three host taxa, irrespective of their specialised chemistry. The few exceptions of highly specialised species that did not overlap between the studied willows included two species of sawflies: *Euura myrtilloides* Kopelke, 1996, which is monophagous on *S. myrtilloides* (Liston et al., 2017) and *Euura munda* (Konow, 1895), which feeds on *S. rosmarinifolia* and *S. starkeana* Willd. (Verzhutskii, 1981). The latter sawfly species has only recently been confirmed in the Czech Republic (J. Macek, pers. observ.). Sawflies generally belong to the most specialised insect herbivores on willows and their host-preferences can be affected by willow specialised chemistry (Kolehmainen et al., 1994; Roininen et al., 1999; Nyman et al., 2006). Similarly, we recorded some narrowly specialised leafhoppers (Hemiptera: Cicadellidae). On *S. repens* and *S. rosmarinifolia*, we recorded *Macropsis impura* (Boheman, 1847) for the first time in the Czech Republic. This species is known as monophagous on *S. repens* and *S. rosmarinifolia* in peaty wetlands in western Europe and dry sandy steppes in eastern Europe, respectively. Only occasionally was it also collected on low-growing individuals of *S. aurita* (Tishechkin, 2002; Nickel, 2003).

In addition to the few highly specialised herbivore species, there were also many Coleoptera and Lepidoptera species for which host chemistry probably did not play a major role (Topp et al., 2002; Volf et al., 2015b). Their presence on the studied willows is likely explained by other factors such as willow leaf morphology or habitat preferences (Volf et al., 2015a). For example, this was probably the case of the leaf-beetle *Cryptocephalus decemmaculatus* (Linnaeus, 1758) that we found on *S. myrtilloides*. This species typically occurs in wet meadows and swamps and it is threatened by habitat loss due to the drying of swamps and peat bogs (Burgess, 2020). While feeding on various willow species, it is considered endangered (EN) on the Czech Red List of invertebrates (Hejda et al., 2017). Similarly, the moth *Anacamptis temerella* (Lienig & Zeller, 1846), that is considered a new species for the Czech Republic (Šumpich et al., in press), is capable to feed on willow species other than *S. myrtilloides* and *S. repens* (Emmet, 1979). Its distribution is thus probably not limited by the scarcity of potential hosts. This was likely also the case of the weevil-beetle *Polydrusus corruscus* E.F. Germar, 1824, considered near threatened (NT), that we found on *S. rosmarinifolia*. The biology of this species is mostly unknown, but it is reported from various willows and hazelnuts (Burakowski et al., 1993).

In conclusion, our results suggest that salicinoids may be responsible for the low abundance of generalists but not of Salicaceae specialists. This provides indirect evidence that for the studied willow species, salicinoids do not serve as an efficient defence against Salicaceae specialists, which is similar to the results of previous studies from other willow species (Volf et al., 2015a). Additionally, despite the differences in specialised chemistry and generalist abundance, specialised chemistry is probably not the primary driver of herbivore community composition in *S. myrtilloides*, *S. repens*, and *S. rosmarinifolia*. We propose that the importance of these willows as hosts of specific and sometimes threatened insect herbivores may primarily result from shared habitat preferences or history between insect herbivores and/or their willow hosts. All three willow taxa typically occur in Central Europe in mesotrophic wetlands, edges of peat bogs or wet meadows (Skvortsov, 1999). These open habitats, however, are threatened by the absence of natural disturbance and changes in land use, including fragmentation and drying of the substrate (Bakker & Berendse, 1999). Our conclusions are limited by the small sample size, which prevented us from testing some hypotheses in a broader context. Yet our results support the findings of other, and typically much broader, studies that highlight the importance of habitats such as peat bogs and mesotrophic wetlands for multiple insect taxa (Spitzer et al., 1999; Spitzer & Danks, 2006; Burgess, 2020). With the exception of a few monophagous specialists, many of the herbivorous insects we found are probably able to feed on other willow species as well, as long as they grow in suitable peat-bog or wet-meadow habitats.

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Table S1. Sites sampled for insect herbivores.

Locality short	Site	Species	GPS coordinates
MYR_MAR	West Bohemia, Prameny, NPP Upolínová louka pod Křížky	<i>S. myrtilloides</i>	N50°4.0913' E12°44.746'
MYR_LUH	South Bohemia, Chlum, Luh u Volar	<i>S. myrtilloides</i>	N48°51.610' E13°53.924'
MYR_LUH	South Bohemia, Chlum, Luh u Volar	<i>S. myrtilloides</i>	N48°51.686' E13°53.926'
MYR_LUH	South Bohemia, Chlum, Luh u Volar	<i>S. myrtilloides</i>	N48°51.679' E13°54.045'
REP_LES	West Bohemia, Lesná, NPP Na požárech	<i>S. repens</i>	N49°42.074' E12°28.144'
REP_LES	West Bohemia, Lesná, NPP Na požárech	<i>S. repens</i>	N49°42.111' E12°27.568'
REP_HUT	West Bohemia, Stará Knížecí Huť	<i>S. repens</i>	N49°42.830' E12°26.485'
REP_HUT	West Bohemia, Stará Knížecí Huť	<i>S. repens</i>	N49°42.829' E12°26.493'
REP_HAJ	Bohemian-Moravian Highlands, Háj u Horního Rápotic	<i>S. repens</i>	N49°34.948' E15°18.399'
REP_HUH	West Bohemia, Stará Knížecí Huť (hranice)	<i>S. repens</i>	N49°42.845' E12°26.485'
REP_PAV	West Bohemia, Pavlův studenec	<i>S. repens</i>	N49°47.858' E12°28.339'
REP_PLE	West Bohemia, Pleš, PP Veský Mlýn	<i>S. repens</i>	N49°32.146' E12°35.880'
REP_POZ	West Bohemia, Lesná, NPP Na požárech	<i>S. repens</i>	N49°42.055' E12°28.128'
REP_ZEH	West Bohemia, Železná u Smolova, (hranice)	<i>S. repens</i>	N49°35.571' E12°34.415'
REP_ZEU	West Bohemia, Železná u Smolova	<i>S. repens</i>	N49°35.526' E12°36.724'
ROS_OHR	South Bohemia, Ohrazení, NP Ohrazení	<i>S. rosmarinifolia</i>	N48°56.923' E14°37.751'
ROS_DEK	South Bohemia, Milíkovice, NP Děkanec	<i>S. rosmarinifolia</i>	N48°51.978' E14°26.451'
ROS_DEK	South Bohemia, Milíkovice, NP Děkanec	<i>S. rosmarinifolia</i>	N48°51.974' E14°26.459'
ROS_DEK	South Bohemia, Milíkovice, NP Děkanec	<i>S. rosmarinifolia</i>	N48°52.004' E14°26.433'
ROS_DEK	South Bohemia, Milíkovice, NP Děkanec	<i>S. rosmarinifolia</i>	N48°51.998' E14°26.417'
ROS_STE	West Bohemia, Vadkov, PP Štěrbů louka	<i>S. rosmarinifolia</i>	N48°58.546' E14°8.422'

Table S2. The number of individuals of insect herbivores recorded on the three studied willow species.

Insect group	Family	Taxon	Host			
			<i>S. myrtilloides</i>	<i>S. repens</i>	<i>S. rosmarinifolia</i>	
Coleoptera	Attelabidae	<i>Temnocerus coeruleus</i> (Fabricius, 1798)	0	0	2	
	Buprestidae	<i>Agrilus viridis</i> (Linnaeus, 1758)	1	0	0	
		<i>Trachys minutus</i> (Linnaeus, 1758)	0	3	2	
	Curculionidae	<i>Acalyptus carpini</i> (Fabricius, 1792)	0	2	0	
		<i>Archarius salicivorus</i> (Paykull, 1792)	1	0	2	
		<i>Phyllobius argentatus</i> (Linnaeus, 1758)	0	0	1	
		<i>Phyllobius pomaceus</i> Gyllenhal, 1834	0	0	4	
		<i>Phyllobius pyri</i> (Linnaeus, 1758)	0	0	12	
		<i>Phyllobius scutellaris</i> L. Redtenbacher, 1849	0	1	0	
		<i>Phyllobius vespertinus</i> (Fabricius, 1792)	0	0	2	
		<i>Phyllobius virideaeris</i> (Laicharting, 1781)	1	0	0	
		<i>Phyllobius viridicollis</i> (Fabricius, 1792)	0	15	47	
		<i>Polydrusus amoenus</i> (Germar, 1824)	0	1	0	
		<i>Polydrusus corruscus</i> Germar, 1824	0	0	1	
		<i>Polydrusus impar</i> Des Gozis, 1882	0	0	1	
		<i>Rhamphus pulicarius</i> (Herbst, 1795)	0	0	2	
		Chrysomelidae	<i>Crepidodera aurata</i> (Marshall, 1802)	0	8	40
			<i>Crepidodera aurea</i> (Geoffroy, 1785)	1	20	5
			<i>Crepidodera fulvicornis</i> (Fabricius, 1792)	3	87	14
			<i>Cryptocephalus decemmaculatus</i> (Linnaeus, 1758)	2	0	0
	<i>Galerucella lineola</i> (Fabricius, 1781)		0	3	0	
	<i>Chrysomela populi</i> Linnaeus, 1758		0	38	11	
	<i>Chrysomela tremulae</i> Fabricius, 1787		0	2	0	
	<i>Chrysomela vigintipunctata</i> (Scopoli, 1763)		0	1	0	
	<i>Lochmaea capreae</i> (Linnaeus, 1758)		52	34	0	
	<i>Phratora laticollis</i> Suffrian, 1851		0	2	0	
	Scarabaeidae	<i>Phyllopertha horticola</i> (Linnaeus, 1758)	1	1	0	
		<i>Agonopterix conterminella</i> (Zeller, 1839)	0	1	0	
	Elachistidae	<i>Agonopterix ocellana</i> (Fabricius, 1775)	1	1	0	
		<i>Arctia caja</i> (Linnaeus, 1758)	0	0	4	
	Erebidae	<i>Rhyparia purpurata</i> (Linnaeus, 1758)	0	0	1	
		<i>Lymantria dispar</i> (Linnaeus, 1758)	0	0	1	
	Gelechiidae	<i>Anacampsis populella</i> (Clerck, 1759)	2	0	0	
		<i>Anacampsis temerella</i> (Lienig & Zeller, 1846)	1	6	0	
		<i>Gelechia sororcullella</i> (Hübner, 1817)	1	0	0	
	Geometridae	<i>Operophtera brumata</i> (Linnaeus, 1758)	0	0	2	
		<i>Selenia dentaria</i> (Fabricius, 1775)	0	1	0	
Lypusidae	<i>Diurnea lipsiella</i> (Denis & Schiffermüller, 1775)	0	2	0		
Noctuidae	<i>Acronicta auricoma</i> (Denis & Schiffermüller, 1775)	0	1	0		
	<i>Agrochola litura</i> (Linnaeus, 1761)	0	0	1		
	<i>Achrochola lota</i> (Clerck, 1759)	2	0	0		
	<i>Amphipyra berbera</i> Rungs, 1949	0	0	1		
	<i>Amphipyra pyramidea</i> (Linnaeus, 1758)	0	0	3		
	<i>Orthosia incerta</i> (Hufnagel, 1766)	0	1	0		
	Notodontidae	<i>Cerura vinula</i> (Linnaeus, 1758)	0	1	0	
		<i>Clostera pigra</i> (Hufnagel, 1766)	1	7	0	
		<i>Acleris hastiana</i> (Linnaeus, 1758)	0	3	2	
	Tortricidae	<i>Gelechia sororcullella</i> (Hübner, 1817)	0	1	0	
		<i>Hedya salicella</i> (Linnaeus, 1758)	1	0	0	
		<i>Pammene populana</i> (Fabricius, 1787)	0	1	0	
		<i>Pandemis heparana</i> (Denis & Schiffermüller, 1775)	1	0	0	
		<i>Ptycholoma lecheana</i> (Linnaeus, 1758)	0	2	0	
		<i>Amauronematus histrio</i> group Konow, 1890	1	1	0	
Symphyta	<i>Amauronematus mundus</i> Konow, 1895	0	0	37		
	<i>Amauronematus viduatus</i> (Zetterstedt, 1838)	24	3	5		
	<i>Amauronematus vittatus</i> (Lepeletier, 1823)	0	0	5		
	<i>Euura myrtilloides</i> Kopelke, 1996	13	0	0		
	<i>Nematus bergmanni</i> Dahlbom, 1835	0	0	1		
	<i>Tenthredo velox</i> Fabricius, 1798	0	1	0		
	<i>Aphrophora pectoralis</i> Matsumura, 1903	0	34	1		
Auchenorrhyncha	Cicadellidae	<i>Hebata vitis</i> (Göthe, 1875)	0	0	3	
		<i>Idiocerus lituratus</i> (Fallén, 1806)	0	3	1	
		<i>Idiocerus</i> sp. Lewis, 1834	0	46	4	
		<i>Kybos</i> sp. Fieber, 1866	0	0	1	
		<i>Macropsis impura</i> (Boheman, 1847)	0	61	75	
		<i>Macropsis prasina</i> (Boheman, 1852)	0	15	0	
		<i>Speudotettix subfuscus</i> (Fallén, 1806)	0	0	1	
Sternorrhyncha	Cixiidae	<i>Cixius similis</i> Kirschbaum, 1868	0	5	0	
		<i>Cavariella pastinacae</i> (Linnaeus, 1758)	1	0	5	
		<i>Chaitophorus populialbae</i> (Boyer de Fons., 1841)	3	6	13	
Aphididae	<i>Pterocomma rufipes</i> (Hartig, 1841)	0	0	2		
	<i>Cacopsylla ambigua</i> (Foerster, 1848)	0	0	2		
	<i>Cacopsylla pulchra</i> (Zetterstedt, 1838)	0	2	273		