

The effect of willow diversity on insect herbivory and predation

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- Abstract**
- 1 The relationship between plant diversity and ecosystems functions such as regulation of insect herbivory is complex and context-dependent. The empirical data necessary to account for these interactions are scarce.
 - 2 Our objective is to test the top-down effects of predation and bottom-up effects of plant traits to understand what drives patterns of herbivory in managed systems.
 - 3 We established single- and multiple-variety willow field plots with up to four varieties, to study herbivory and predation, and greenhouse experiments to investigate the effects on insect preference and performance.
 - 4 Herbivore damage in the field was higher in single- as opposed to multiple-variety plots for the two most prevalent insect types in one of the 3 years. Predation was higher in multiple-variety plots than in pure plots during 2016 but the opposite occurred in 2018. Predation on leaf beetle eggs was higher in multiple-variety plots, but the result was related to specific variety mixtures rather than increased numbers of varieties. Leaf beetle feeding choice and egg-laying were influenced to some extent by plant diversity.
 - 5 Our results give insights into the links between plant diversity, composition and levels of herbivore damage. They also spark questions about how temporal aspects influence these links.

Keywords Intraspecific variation, perennial plants, *Phratora vulgatissima*, plant mixtures, plant–insect interactions, *Salix*, willow.

Introduction

Biodiversification of agricultural and forest fields by increasing crop diversity is a step closer to achieve global goals for sustainable development. Plant species richness increases below- and above-ground biodiversity (Scherber *et al.*, 2010) and has substantial effects on ecosystem functions and services (Crutsinger *et al.*, 2006; Hughes *et al.*, 2008; Tilman *et al.*, 2014; Dainese *et al.*, 2019; Jactel *et al.*, 2021). A diverse ecosystem is also known to be more resilient to human disturbance and natural events in many cases (McCann, 2000; Oliver *et al.*, 2015).

Today, agriculture and forestry suffer enormous economic costs due to herbivorous insects causing production losses. Increasing crop diversity, and thereby biodiversity, while at the same time reducing problems related to pests and diseases is a tempting way forward. Species diversity and genetic diversity of plant communities have significant effects on consumer communities (Abdala-Roberts *et al.*, 2015; Koricheva & Hayes, 2018; Barantal *et al.*, 2019). However, their relative importance differs

among studies and the results reflect the traits of the specific system studied, for example, the phylogenetic distance of the plants included and the host specificity of the herbivores. Despite recent intensified focus on the effects of plant genetic diversity on ecosystem processes such as damage caused by insect herbivores (Schuldt *et al.*, 2010; Ebeling *et al.*, 2014; Koricheva & Hayes, 2018; Jactel *et al.*, 2021), there are few studies that provide the evidence needed to understand mechanisms behind the sometimes varying results obtained (Whitham *et al.*, 2006; Cardinale *et al.*, 2012; Staab & Schuldt, 2020; Weih *et al.*, 2020; Jactel *et al.*, 2021). In particular, there is a knowledge gap regarding how plant diversity affects the processes involved in trophic interactions (Ebeling *et al.*, 2014; Weih *et al.*, 2019; Staab & Schuldt, 2020).

Recent work shows that systems with more natural enemies exert a stronger top-down pressure, which results in less damage caused by insect herbivores (Oliver *et al.*, 2015). To find how patterns of herbivory relate to plant community heterogeneity, we need a better understanding of how herbivorous insects and their predators interact with their immediate surroundings. One explanation is that diverse plant systems create available niches that make room for more diverse insect communities

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(Hunter & Price, 1992). A contrasting explanation, in accordance with the resource concentration hypothesis (Root, 1973), is that as an effect of more niches, the size of specific niches decreases (given a specific area) and the number of insects per type should also decrease. Yet another scenario, referred to as the resource dilution effect (Otway *et al.*, 2005), is that the damage per plant in diverse systems increases because there are relatively fewer available hosts. Recent studies, however, suggest that it is not the diversity *per se* that affects insect damage in mixed stands, instead the important factor is the composition of species; the larger the phylogenetic distance, the stronger effect (Dinnage, 2013; Castagneyrol *et al.*, 2014).

Yet another proposed hypothesis for herbivore suppression in diverse plant habitats is that herbivory depends on the identity of neighbouring plants, the so-called associational effects (Barbosa *et al.*, 2009). Direct associational effects can occur when neighbouring plants affect herbivore behaviour because diverse plant stands create challenges for insects to locate host plants (Bernays, 2001). Studies indicate that herbivores in diverse plant stands move more as compared with those in monocultures (Cantelo & Sanford, 1984; Power, 1988, 1991; Aluja *et al.*, 1997) and probably spend less time feeding. Specific plant compositions may decrease (Underwood *et al.*, 2014) or increase (Barbosa *et al.*, 2009) the likelihood of detection of focal plants by herbivores, and localization of 'the best' host plant becomes more difficult in a diverse habitat.

Indirect associational effects of neighbours can affect herbivores through altered plant traits (Bailey *et al.*, 2014). The proposed mechanism is that through niche differentiation and effective use of available resources, plants grown in diverse communities express traits differently compared with the same plant grown in monocultures. Examples of this include plants grown in mixed cultures that have different leaf nitrogen content and/or leaf areas compared with the same plants grown in pure cultures (Kos *et al.*, 2015; Hoeber *et al.*, 2017; Weih *et al.*, 2021).

We explore the above-mentioned mechanisms in the context of improving ecosystem function through genetic diversity. Using a range of varieties within a plant genus has been suggested by Tooker and Frank (2012) to be a particularly suitable approach in this context. We use willows (*Salix*) as a model system because there are plenty of knowledge generated in studies of plant–plant and plant–insect interactions using this genus (Weih *et al.*, 2019). Willows are easily propagated from equal-sized cuttings, making controlled ecological studies possible. They are rapid growers, and the results from willow model systems can serve as representatives for other fast-growing woody plants. In addition, willows have an array of plant defences, herbivores and natural enemies (Glynn & Larsson, 2000; Björkman *et al.*, 2004; Björkman & Eklund, 2006). Willows are grown for renewable energy (Verwijst *et al.*, 2013; Weih *et al.*, 2020) and bioremediation (Dimitriou & Aronsson, 2005) in short rotation coppice. The coppicing system provides a spatio-temporally dynamic habitat, which influences biodiversity over time (Vanbeveren & Ceulemans, 2019). Occasionally plantations can be severely damaged by leaf beetles. The most common beetle in the area of our study is *Phratora vulgatissima* (Coleoptera: Chrysomelidae), which during an outbreak can cause plant growth reductions with up to 40% (Björkman *et al.*, 2000).

Our aim is to test the effect of plant diversity on insect damage and understand the underlying mechanisms, such as top-down effects (predation) and bottom-up effects (plant traits) on herbivory. We do so in a series of field and greenhouse experiments where willow varieties were grown in pure or mixed cultures. We predict that:

- 1 As a result of top-down and bottom-up effects combined, we expect that plant damage will differ in plots where willows are grown in mixtures compared with pure cultures. We predict that plant damage will differ between specific willow varieties and that there will be significant interactions between the willow variety and the variety composition of the stands (e.g. due to resource concentration/dilution effects).
- 2 Predation has been suggested to be the driving mechanism in reducing damage in mixed plots. We therefore predict that predation pressure will be different in plots where willow varieties are grown in mixtures as compared with pure cultures, and that predation on individual willow varieties will be affected by the plot composition (e.g. due to resource concentration/dilution effects).
- 3 Direct associational effects acting on insect behaviour are suggested to cause reduced damage in mixed cultures because a diverse plant community may confuse the herbivore when choosing its host. Therefore, we predict that the leaf-chewing insect (*P. vulgatissima*) in our study will feed less on a specific variety when it is grouped with other varieties compared with when it is grouped with plants of its own variety.
- 4 Associational effects may also act indirectly on insect damage due to differences in plant traits in mixed cultures as compared with the traits of plants grown in pure cultures. We predict that in the controlled conditions of our experiment, there will be differences in adult feeding, egg-laying and larval performance of a leaf-chewing insect (*P. vulgatissima*) on willow varieties grown in mixtures as compared with varieties grown in pure cultures.

Material and methods

Field study site

The willow study site is planted on agricultural land in 2014 and follows an ordinary/commercial management regime with harvest/coppicing every third year (coppiced in the winter of 2016/2017). It is located in Uppsala, central Sweden (59.8174°N, 17.6575°E), and is a part of the Ecolink-Salix project described by Hoeber *et al.* (2018). The Ecolink-Salix design uses the willow model system to assess factors that affect biomass production in relation to variety identity and diversity (Weih *et al.*, 2019). The willow varieties used are described in Weih and Nordh (2002, 2005): 'Björn' (*Salix schwerinii* E. Wolf. × *Salix viminalis* L.), 'Jorr' (*S. viminalis* L.), 'Loden' (*Salix dasyclados* Wimm.), and 'Tora' (*S. schwerinii* E. Wolf. × *S. viminalis* L.). 'Björn' and 'Tora' are full siblings; 'Jorr' is closely related taxonomically to 'Björn' and 'Tora'; whereas 'Loden' belongs to a different species and thus is taxonomically most distant from the other three varieties (Larsson, 1998).

The willow field consists of three replicates, each containing 15 plots with four diversity levels: four plots of pure cultures

9.6 m

D 33	ABCD 34	AB 39	AC 40	ACD 45	Rep III	28.8m	9.6 m
ABD 32	AD 35	ABC 38	B 41	CD 44			
BC 31	BD 36	A 37	BCD 42	C 43			
AD 18	ABCD 19	CD 24	AB 25	ACD 30	Rep II	28.8m	86.4 m
BCD 17	C 20	ABC 23	ABD 26	BD 29			
BC 16	AC 21	B 22	D 27	A 28			
ABD 03	D 04	C 09	CD 10	BCD 15	Rep I	28.8m	
BC 02	A 05	AC 08	ABC 11	AD 14			
B 01	AB 06	ACD 07	BD 12	ABCD 13			

Figure 1 The map of the field experiment located at Ultuna, SLU campus. Twelve of the 15 plots in each replicate were sampled for herbivory and predation indices: four single-variety plots and eight multiple-variety plots. There are 144 willows (*Salix* spp.) in each plot, spacing is approx. 0.8 m on all sides. Letters refer to the different varieties; A = 'Björn', B = 'Jorr', C = 'Loden' and D = 'Tora'. Selection of plants was made with a random number generator, stratified for each willow variety in the plots. In single-variety plots, 10 plants were randomly sampled; in two-variety plots, five plants of each of the two varieties were randomly sampled; in three-variety plots, three plants of each willow variety were randomly sampled plus one extra draw; in the four-variety plots, three plants were sampled for two of the varieties in the plot and two plants were sampled for the two other varieties.

('Björn', 'Jorr', 'Loden' or 'Tora'), six plots contain two varieties, four plots have three varieties and one plot has all four varieties (Fig. 1). We sampled 12 of the 15 plots per replicate and 10 willows per plot in 2016, 2017 and 2018. In each replicate, all four plots of pure culture, five of the two-variety plots, two of the three-variety plots and the four-variety plot were sampled. Within this sampling scheme, plots were randomly selected to represent the various combinations of willow varieties.

Comparing herbivory levels in the field

Herbivory data were collected in 2016 on un-coppiced willows of 3-year-old root stocks in their third year of growth (mean heights

2.7–3.5 m); in 2017, willows of 4-year-old root stocks in their first year of re-growth after being coppiced the previous winter (mean heights 1.7–2.7 m); and in 2018, on willows of 5-year-old root stocks in their second year of re-growth after coppicing (mean heights 3.0–4.3 m). Samples were taken between the last week of September and mid-October each year. In 2016 and 2017, the same 10 individual trees (randomly selected within the plots) were sampled in 12 plots that represented the various mixes in each replicate (see Fig. 1 for plot types and how varieties were selected for sampling). In 2018, we sampled the same varieties and mixtures but different individual trees. This was due to vole and roe deer damage in some of the plots during the preceding winter. Each year 360 willows and 20

leaves per willow were assessed for herbivory damage, making a total of 21 600 leaves for the 3 years of the study. Leaves were individually inspected, and all damage was recorded by category; leaf chewers, leaf skeletonizers, leaf galls, leaf miners, leaf folders and leaf rollers in accordance with the international protocol used in Tree Diversity Network (Jactel *et al.*, 2016) aiming to compare herbivory across ecological zones (Verheyen *et al.*, 2016). The only other kind of damage found on the leaves was necrotic spots that we could not distinguish if it was caused by leaf-sucking insects such as aphids or by fungal infection. For this reason, we did not record this kind of damage.

Sampling of leaves was done according to the protocol by Jactel *et al.* (2016) where herbivore damage is assessed on a predetermined and fixed number of leaves per tree (20 in our experiment). Ten leaves were collected from each of two branches per tree; one from among the tallest shoots and one from the shorter. On both shoots, five fully expanded leaves were randomly chosen in the upper region of the shoot and five leaves closer to the base of the shoot.

Predation experiments in the field

Plasticine caterpillars. In order to estimate predation in willow plots of single and multiple varieties, willow plants that were assessed for herbivore damage also were assessed for predation. In 2018, however, we did not include plots with four varieties. We followed the Howe *et al.* (2009) standardized plasticine caterpillar method in 2016 and 2018. Following the protocol by Low *et al.* (2014), two plasticine caterpillars were placed on each sample tree. One was placed midway on a dominant shoot and the other midway on a smaller shoot. Because willows grow rapidly after coppicing, and different varieties have different growth patterns, there was no single standard height for positioning the model caterpillars. The caterpillars were left in the field, exposed to potential predators for about 10 days in 2016 (Rep 1: 31 May–10 June, Rep 2: 17–27 June, Rep 3: 13–24 June) and a little longer in the cooler 2018 (Rep 1: 18 June–3 July, Rep 2: 20 June–5 July, Rep 3: 25 June–8 July). The caterpillars were exposed to predation for different times and also for different periods, which may complicate comparisons because predators may be differently active due to, for example, temperatures. However, since we aimed at comparing relative numbers between different mixes and the timing was the same within replicates, this should not be a problem. Under a stereo microscope, we recorded the number of marks on each caterpillar that could be attributed to four categories of predators: birds, arthropods, mammals and reptiles. We separated the arthropods into small (less than 2 mm between mandible marks) and large arthropods (greater than 2 mm between mandible marks). No attempt was made to identify the species of predators. We record bite marks only and our data most likely include repeated bites by the same individual predators.

Leaf beetle eggs. Eggs of the leaf beetle *P. vulgatissima* were collected in May 2018 in a *Salix* plantation south of Uppsala. Using the same plots as for the caterpillar experiment described previously, egg batches (range 9–28 eggs, mean 19 eggs) were attached to leaves with a thin insect pin in late May (replicate

1 on 25 May and replicates 2 and 3 on 27 May). Four plants of each variety were selected in all plots and one egg batch was attached on each plant. The eggs were placed at approximately equal distance from the ground (about 1.5 m). Before adding the eggs, the shoots were shaken to remove potential natural enemies that might have been on the shoots. After 5 days, the eggs were removed and examined under a stereo microscope. The integrity of the egg encasement shell is broken on predated eggs, making them easy to distinguish from intact eggs.

Plant and insect material for greenhouse studies

Exactly 5 cm cuttings of the varieties ‘Björn’, ‘Jorr’, ‘Loden’, and ‘Tora’ were planted in pots placed in a greenhouse (temperature 20 °C, L18 : D6, Hasselfors S-soil) in early April 2017. Cuttings of the variety 78 021 (*S. viminalis* L.), known to be suitable food for *Phratora* beetles (Lehrman *et al.*, 2012), were also planted for feeding material for the beetles when they were not used in an experiment. This prevented preconditioning by providing a food source that was not included in the experiments (Björkman *et al.*, 2013). The plants were liberally watered and after 6 weeks they were all fertilized. Genotypic differences in plant traits (plant height, leaf chlorophyll and leaf size) after 6 weeks of growth are described in Supporting information, Table S1.

Overwintering beetles of *P. vulgatissima* were collected from cracks and under the bark of trees (mainly rowans, *Sorbus*) adjacent to a willow field in Ärentuna, approximately 20 km north of Uppsala in April 2017.

Estimating herbivory in greenhouse experiments

To test predictions 3 and 4, two feeding venues were set up in a greenhouse to study insect preference and insect performance in relation to plant diversity. The four varieties of *Salix* described above were either grown singly in pots and later arranged in groups (setting 1) or grown together in containers (setting 2). This was done to be able to separate direct and indirect plant effects on herbivores, because in setting 1 we do not expect changes in plant traits, but in setting 2 the plants interact at root level and need to compete for resources, and it is likely that plant traits may be affected differently in the different mixes. Plants were arranged to replicate the different levels of plant diversity from the field experiments; they were arranged as monocultures with one variety (‘Tora’, ‘Björn’, ‘Loden’ or ‘Jorr’), or as mixtures of two varieties (‘Tora’ and ‘Loden’), mixtures of three varieties (‘Tora’, ‘Loden’ and ‘Jorr’) or as mixtures with all four varieties. The varieties chosen for the mixes were as phylogenetically different as possible. In setting 1, the beetles were free to forage where they chose, whereas in setting 2, one plant was selected to feed one beetle (no-choice experiment). Insect preference was assessed by the area of leaf damaged by adult feeding and by the number of eggs laid. Insect performance was assessed by larval survival and larval development.

Direct effects of single- and multiple-variety groups (setting 1). After 6 weeks of growth, four potted plants were

arranged together in a 2*2 square and were enclosed with a cage constructed of perforated polypropylene bags (Baumann Saatzuchtbedarf). Each treatment was replicated as follows: 32 single-variety groups (12 of 'Björn', 8 of 'Jorr', 4 of 'Loden' and 8 of 'Tora'), 10 two-variety mixes consisting of 'Loden' and 'Tora', 12 three-variety mixes (four replicates consisting of 1 'Jorr'+1 'Loden'+2 'Tora', four replicates of 1 'Jorr'+2 'Loden'+1 'Tora', and four replicates of 2 'Jorr'+1 'Loden'+1 'Tora') and 12 four-variety mixes. The experiment included 66 groups and 264 individually potted plants. The plants within a group were carefully selected to be as similar as possible with respect to size (thereby the different number of replicates per treatment).

In each cage, one male and one female leaf beetle were free to choose were to feed for 4 weeks before they were removed and damage was measured. We calculated the damage by counting the number of leaves with damages out of 15 randomly taken leaves per plant. We also counted all the leaves (damaged and undamaged) on the plant.

Indirect effects of single- and multiple-variety combinations (setting 2). Eight equally spaced cuttings were grown together in a container. Each of the four single varieties and the two-, three- and four-variety mixes were replicated 12 times, in total 84 containers. Because it was not possible to have equal amounts of plants per variety in the containers with 3-mixes, four of the 12 replicates consisted of 2 'Jorr'+3 'Loden'+3 'Tora', four replicates of 3 'Jorr'+2 'Loden'+3 'Tora', and four replicates of 3 'Jorr'+3 'Loden'+2 'Tora'.

In each container, one plant per variety was randomly selected and leaves from the mid-section were collected to assess adult feeding, egg-laying and larval performance. These studies were conducted in a climate chamber (20 °C, 18 h light). One leaf and one female were placed in transparent plastic boxes (21 × 17 × 6 cm), and leaves were replaced every second day for 10 days. Leaf size, number of eggs laid and total leaf area eaten (measured by ImageJ software) were recorded. Larval survival and larval development were followed in a similar manner. A cluster of eggs (mean 8 eggs, range 5–13) from each female was placed in a plastic box on a leaf from the same plant that the female had been feeding on. The leaves were renewed twice a week, and at the same time, the number of eggs and larvae were counted until all the larvae had pupated. Larval survival and development were calculated. Larval development time was calculated as the number of days from when all the eggs in the group had hatched to that all larvae had pupated.

Data analysis

Field data

Herbivory. We assessed the effects of willow diversity on damage caused by six different types of insect herbivores in two ways. We compared insect damage in single-variety plots with multiple-variety (aggregated two, three and four-variety) plots with the Kruskal–Wallis Chi-square tests (PROC NPAR1WAY SAS 9.4). We also compared plots with one, two, three and four varieties per plot using a general linear mixed model with plot

as a random factor (PROC GLIMMIX SAS 9.4 for binomial distribution with Tukey–Kramer adjustments for multiple comparisons). To assess the role of willow variety on levels of herbivory, we used a general linear mixed model with plot as random factor and binomial distribution (PROC GLIMMIX SAS 9.4). In addition, we analysed the effects of willow variety and the interaction with the genotypic composition of the plots (varieties association) on the insect damage levels (PROC GLIMMIX for binomial distribution of the data with plot as random effect and Tukey–Kramer adjustments).

Predation. The role of willow variety on levels of predation was compared with Kruskal–Wallis tests (PROC NPAR1WAY). To assess the effects of willow diversity (comparing single- vs. multiple-variety plots) on predation by arthropods, birds and mammals, as indicated by bite marks on plasticine caterpillars general linear mixed model with block and plot as random factors (PROC GLIMMIX, Poisson distribution). Comparisons of predation levels in plots with one, two, three and four varieties were assessed with a general linear mixed model with plot as a random factor (PROC GLIMMIX Poisson distribution with Tukey–Kramer adjustments for multiple comparisons). Analyses of genotypic composition was conducted by looking at each variety and its interaction with the genotypic composition of the plot in which it grew, with PROC GLIMMIX for Poisson distribution with plot as a random factor and Tukey–Kramer adjustments of multiple comparisons.

Analysis of the effects of willow diversity on predation of leaf beetle eggs was carried out using linear models in R version 3.4.4 (R Core Team, 2020), with willow diversity and variety and their interaction as factors. The number of eggs from the beginning was used as a covariable and replicate was included as a factor to account for random variation in the field. The predation variable was log-transformed to achieve normal distributed residuals.

Greenhouse data

To analyse the effect of willow diversity on herbivory by beetles in setting 1, we used a linear mixed model (lmer in R 3.4.4). Variety and diversity level together with their interaction were included as explanatory factors and group as a random factor because the plants in a group cannot be considered independent. The total number of leaves on a plant was included as a covariate in the full model, but because it did not affect the outcome of the main factors, we removed it from the model.

Beetle herbivory in setting 2 was analysed using separate linear mixed models (lme in R 3.4.4) for each variety with plant ID as a random factor because we had repeated measurements (five leaves from the same plant per female). In the models, we also included a weight variable to achieve homogenous variances and normal distributed residuals. We also included leaf size as a covariate in those models.

In the analyses of egg-laying and larval performance (survival and development time), we used variety, diversity and their interaction as explanatory variables in the full model. We used linear models (lm in R 3.4.4) for egg-laying and larval development and a generalized linear model (glm in R 3.4.4) with a binomial error distribution for larval survival because it was

measured as proportions. Group size was added as a covariate in the analyses of larval survival and development.

Results

Stand-level plant diversity effects on insect damage

We found statistically significant differences in damage when comparing single-variety plots with multiple varieties plots (those with two, three and four varieties pooled) in 2016 for the two most prevalent herbivores; leaf chewers and leaf skeletonizers. Both were higher in single-variety than in multiple-variety plots (Kruskal–Wallis Chi-square test: leaf chewers: $\chi^2 = 6.8$, d.f. = 1, $P = 0.01$ and skeletonizers $\chi^2 = 3.88$, d.f. = 1, $P = 0.05$). No differences were found in other years or for other insect types. Table 1 shows the means (\pm SE) for each insect type and year.

Because leaf-chewing insects were the most prevalent herbivores in the study, we used them to assess if their damage levels were statistically different when comparing plots with one, two, three or four willow varieties. There was no statistically significant difference in any year (2016: $F = 1.36$, d.f. = 3, $P = 0.29$, 2017: $F = 0.50$, d.f. = 3, $P = 0.69$, and 2018: $F = 0.58$, d.f. = 2, $P = 0.57$).

Varieties played a role in the levels of herbivory in 2017 for leaf-chewing insects ($F = 12.69$, d.f. = 3, $P = 0.0001$) but not in the other years (2016: $F = 0.85$, d.f. = 3, $P = 0.47$, 2018: $F = 1.46$, d.f. = 3, $P = 0.23$). ‘Jorr’ had significantly lower levels of damage than the other willow varieties. Sibling varieties ‘Tora’ and ‘Björn’ had the same levels of damage and ‘Loden’

had higher levels than ‘Tora’. Supporting information, Table S2 shows the mean number of leaves (of 20) with damage for each insect type in each year.

We assessed if willow variety in relation to the composition of the plot (i.e. the interaction between variety and composition) had an effect on damage caused by leaf chewers. Analyses revealed no statistically significant effects in 2016 or 2018 (2016: $F = 0.80$, d.f. = 24, $P = 0.71$ and for 2018: $F = 61.58$, d.f. = 21, $P = 0.32$). In 2017, the model indicated effects of varietal composition ($F = 31.45$, d.f. = 24, $P = 0.01$) but after Tukey–Kramer multiple comparisons adjustment, there was no statistically significant difference.

Stand-level plant diversity effects on predation

Variety diversity affected the levels of predation, but the results varied between predator types and years (Table 2). In 2016, we found that the total number of bite marks (from large and small arthropods together) were higher in the multiple-variety plots (plots with two, three and four varieties pooled) (mean 14.76, SE 1.18 bite marks per tree) as compared with single-variety plots (mean 9.37, SE 1.03) ($F = 3.35$, d.f. = 1, $P = 0.003$). In 2018, the results were the opposite; the total number of bite marks were higher in the single-variety plots (mean = 4.86, SE = 0.85) than in multiple-variety plots (mean = 2.84, SE 0.36) ($F = 6.42$, d.f. = 1, $P = 0.02$). In 2016, there were no mammal bite marks and no differences between levels of bird bite marks ($F = 0.06$, d.f. = 35.12, $P = 0.82$). In 2018, there were no differences

Table 1 Mean (SD) number of leaves (of 20) with damage by six types of insect herbivores (leaf chewers, leaf miners, leaf galls, leaf skeletonizers, leaf rollers and leaf tiers) in plots with a single variety and plots with mixtures of two, three and four willow varieties. In 2016 and 2018, there were no leaf miners; in 2017 and 2018, there were no leaf galls; in 2016 and 2017, there were no leaf tiers; in 2018, no four-variety plots were sampled

Leaf-herbivore type	2016		2017		2018	
	Single-variety mean (SD)	Multiple-variety mean (SD)	Single-variety mean (SD)	Multiple-variety mean (SD)	Single-variety mean (SD)	Multiple-variety mean (SD)
Chewer 1	2.99 (0.85)		10.1 (3.7)		6.7 (3.1)	
Chewer 2-mix		2.9 (0.7)		9.8 (3.1)		7.3 (3.1)
Chewer 3-mix		2.6 (0.7)		12.1 (10.9)		7.7 (2.7)
Chewer 4-mix		2.7 (0.65)		9.6 (3.5)		—
Miner 1	0.02 (0.15)		0.15 (0.42)		0	
Miner 2 mix		0.02 (0.13)		0		0
Miner 3 mix		0.17 (0.31)		0.13 (0.5)		0
Miner 4 mix		0		0.1 (0.3)		—
Galler 1	0.31 (0.5)		0.016 (0.1)		0.35 (0.3)	
Galler 2-mix		0.39 (0.5)		0		0.02 (0.2)
Galler 3-mix		0.19 (0.39)		0.01 (0.3)		0.02 (0.1)
Galler 4-mix		0.33 (0.5)		0		—
Skeletonizer 1	1.69 (0.92)		1.0 (1.6)		4.5 (4.3)	
Skel. 2-mix		1.59 (0.82)		0.7 (0.4)		4.0 (3.7)
Skel. 3-mix		1.3 (0.6)		0.93 (1.8)		3.4 (3.3)
Skel. 4-mix		1.7 (0.7)		1.2 (1.8)		—
Roller 1	0.11 (0.3)		0.28 (0.7)		0.06 (0.23)	
Roller 2-mix		0.05 (0.22)		0.1 (0.4)		0.12 (0.4)
Roller 3-mix		0.24 (0.4)		0.11 (0.3)		0.06 (0.2)
Roller 4-mix		0.23 (0.4)		0.17 (0.4)		—
Tier 1	0		0		0.5 (0.2)	
Tier 2-mix		0		0		0.05 (0.2)

Table 2 Mean number of bite marks of small arthropods (<2 mm), large arthropods (>2 mm), birds and mammals on plasticine caterpillar placed on willows in single-variety, two-variety, three-variety and four-variety willow plots during 2016 and 2018. In 2016: One-variety plots $n = 90$ willows, two-variety plots $n = 60$ willows, three-variety plots $n = 60$ willows and four-variety plots $n = 30$ willows. In 2018: One-variety plots $n = 41$ willows, two-variety plots $n = 120$ willows, three-variety plots $n = 50$ willows, and there were no four-variety plots. Two plasticine caterpillars were sampled on each willow. Bold-faced comparisons indicate a statistically significant difference when comparing single- and multiple-variety plots

Type of willow plot	Small arthropods		Large arthropods		Birds		Mammals	
	2016	2018	2016	2018	2016	2018	2016	2018
Single-variety	3.45 (0.31)	0.67 (0.14)	1.39 (0.22)	1.65 (0.21)	0.18 (0.08)	0.28 (0.11)	0.00	0.012 (0.01)
Multiple-variety aggregated	5.18 (0.23)	0.49 (0.07)	2.41 (0.17)	1.13 (0.10)	0.19 (0.06)	0.44 (0.05)	0.02 (0.01)	0.008 (0.01)
Single-variety	3.45 (0.30)	0.67 (0.14)	1.39 (0.22)	1.65 (0.21)	0.18 (0.09)	0.28 (0.11)	0.012 (0.01)	0.001 (0.01)
Two-variety	4.02 (0.36)	0.44 (0.08)	2.91 (0.27)	1.17 (0.23)	0.25 (0.11)	0.42 (0.06)	0.008 (0.01)	0.01 (0.01)
Three-variety	6.14 (0.36)	0.61 (0.12)	1.98 (0.27)	1.04 (0.21)	0.05 (0.11)	0.48 (0.10)	0.25 (0.01)	0
Four-variety	5.57 (0.51)	—	2.3 (0.38)	—	0.33 (0.15)	—	0	—
Total nr. bite marks on caterpillars	2156	221	967	520	88	172	3	3

between the levels of bird or mammal bite marks ($F = 2.0$, d.f. = 1, $P = 0.16$, and $F = 0.20$, d.f. = 1, $P = 0.67$ respectively).

Separate analyses of the two types of arthropods in each year revealed that in 2016, the number of bite marks from large arthropods ($F = 5.73$, d.f. = 1, $P = 0.02$) and small arthropods ($F = 5.73$, d.f. = 1, $P = 0.03$) were higher in mixed plots as compared with single-variety plots. In 2018, there were more bite marks from large arthropods in single-variety plots than in multiple-variety plots ($F = 5.04$, d.f. = 1, $P = 0.03$), but there was no difference between single- and multiple-variety plots for small arthropods ($F = 1.98$, d.f. = 1, $P = 0.16$). Mean values for comparisons are in Table 2.

When comparing predation in plots with one, two, three and four varieties, we found that there were no statistically significant differences in levels of predation by large or small arthropods in either year. However, when combining the two types of arthropods together, there was a significant difference in 2018 ($F = 3.34$, d.f. = 2, $P = 0.05$), but not in 2016. The comparisons with Tukey–Kramer adjustments showed that in 2018, predation was higher in single varieties as compared with plots with two varieties. The mean number of bite marks is found in Table 2.

The results from the field predation experiment on *Phratora* eggs revealed that variety diversity affected the number of eggs predated (Fig. 2). Predation was 43% higher on egg batches in plots with three varieties compared with single-variety plots (t -value = 2.0, d.f. = 181, $P = 0.04$). There were, however, no differences between plots with one and two varieties (t -value = 0.92, d.f. = 181, $P = 0.36$).

Plasticine caterpillars on certain willow varieties experienced more predation than on others. In 2016, but not in 2018, there were significantly more bite marks from large arthropods on caterpillars of the varieties ‘Jorr’ and ‘Loden’ than on ‘Björn’ or ‘Tora’ ($\chi^2 = 13.23$, d.f. = 3, $P = 0.004$). No variety effect was found on the number of bite marks caused by smaller arthropods, birds or mammals in 2016 or 2018. Nor did variety influence predation on egg batches ($F = 1.10$, d.f. = 180, $P = 0.35$).

In 2016, stand composition interacted with variety on the effect of predation by arthropods (small and large bites aggregated) ($F = 5.75$, d.f. = 10, $P = 0.001$). Analyses of the multiple comparisons with Tukey–Kramer correction revealed one statistically significant difference: ‘Tora’ had less predation by

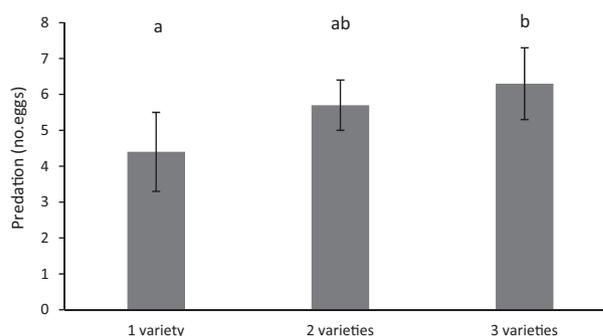


Figure 2 Number of eggs (mean \pm SE) predated on *Phratora vulgatissima* eggs in field plots of either single varieties, two varieties or three varieties of the willow varieties ‘Björn’, ‘Jorr’, ‘Loden’ and ‘Tora’ in 2018. Batches of eggs (range 9–28 eggs, mean 19) were attached to four willow shoots per plot and exposed to predators for 5 days. Letters above bars show statistical significance.

arthropods in single-variety plots (mean = 8.63, SE = 5.26) as compared with ‘Tora’ in mixed plots with ‘Jorr’ and ‘Tora’ (mean = 30.28, SE = 5.26) ($F = 5.28$, d.f. = 13, $P = 0.015$) (Fig. 3a). In 2018, there were no significant differences in level of predation by arthropods among plots with different variety compositions ($F = 2.12$, d.f. = 11, $P = 0.07$). There were, however differences among varieties ($F = 4.35$, d.f. = 3, $P = 0.006$) and for the interaction between plot composition and variety ($F = 2.77$, d.f. = 8, $P = 0.006$). Analyses of the multiple comparisons among varieties and plot composition revealed no significant differences (Fig. 3b). There were no significant differences in either year with regard to predation by birds or mammals.

When comparing specific variety combinations with regard to egg predation, predation increased in some mixed plots compared with the single-variety plots, but not in others (Fig. 3c). Of the variety combinations tested, egg predation was higher in the plots with mixes of ‘Björn’ and ‘Loden’; and in the mixes with ‘Björn’, ‘Jorr’ and ‘Loden’ than in plots with ‘Björn’ only (t -value = 1.90, d.f. = 172, $P = 0.06$ and t -value = 2.27, d.f. = 172, $P = 0.007$ respectively).

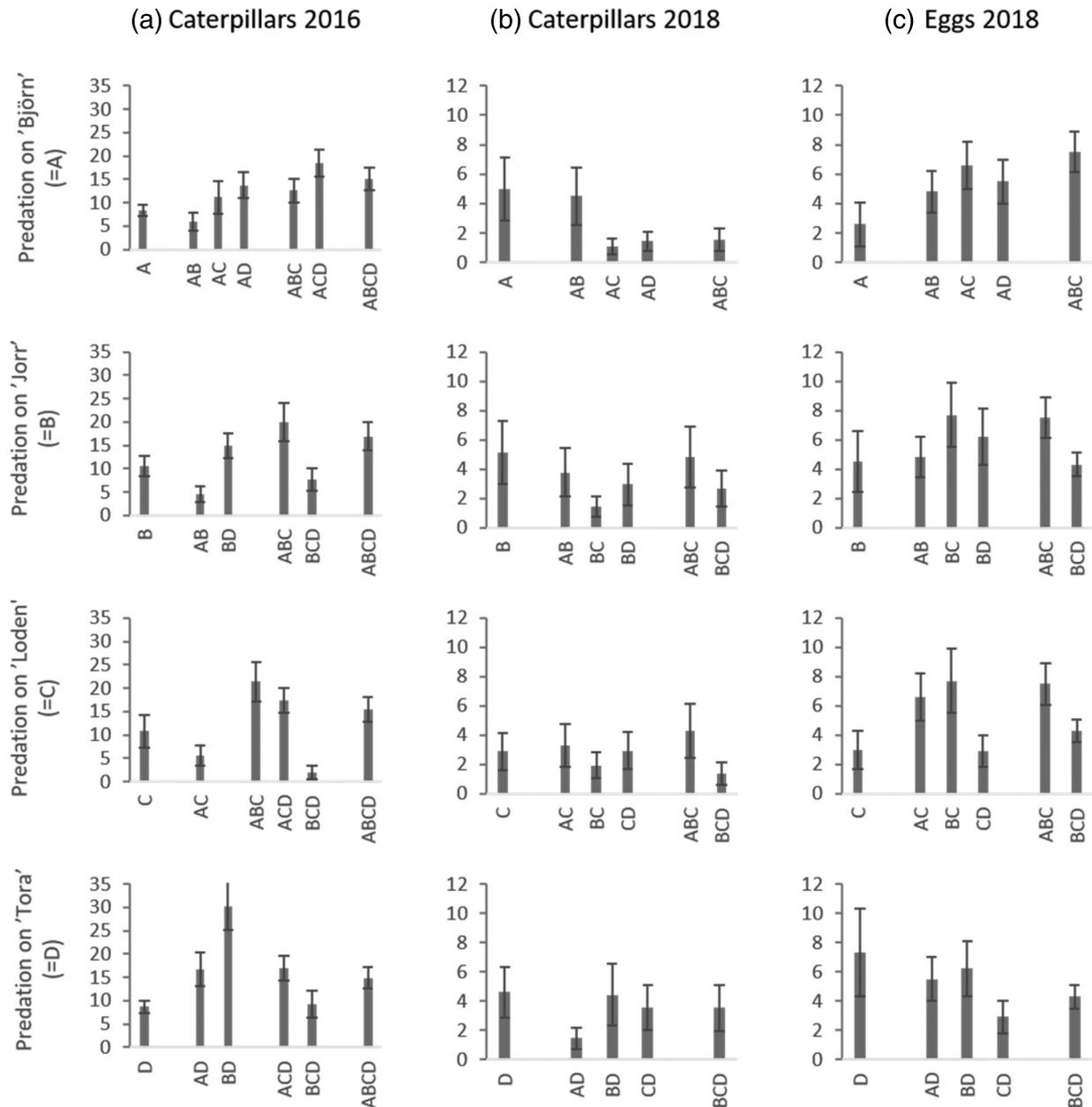


Figure 3 Predation on *Salix* varieties, either 'Björn' (=A), 'Jorr' (=B), 'Loden' (=C), or 'Tora' (=D), in plots with different variety combinations in a willow field experiment. (a) Number of bite marks on plasticine caterpillars in 2016, (b) number of bite marks on plasticine caterpillars in 2018 and (c) number of eggs of a leaf beetle (*Phratora vulgatissima*) predated in 2018. Bars show means \pm 1 SE.

Effects of plant diversity on feeding in the choice experiment

A greenhouse experiment addressed the feeding preferences of beetles in two settings. When beetles were placed on plants in groups consisting of either 1, 2, 3 or 4 varieties (setting 1), plant diversity had no effect on damage as measured by the number of leaves eaten (of 15) per plant ($F = 0.93$, d.f. = 45, $P = 0.44$). Adult beetles fed on 6.8–7.8 leaves in average. Variety in combination with plant diversity, however, affected adult feeding. Fewer leaves were eaten on the varieties 'Loden' and 'Björn' in single-variety groups but significantly more leaves were eaten on 'Loden' in 3-mixes (t -value = 2.12, d.f. = 73.52,

$P = 0.04$) and 'Björn' in the 4-mix (t -value = 2.60, d.f. = 100.37, $P = 0.01$) (Fig. 4a).

Effects of plant diversity on feeding, egg-laying and larval performance in the no-choice experiment

In the second feeding experiment, beetles were given only one of the four varieties (setting 2). 'Loden' had the least amount of feeding, regardless of the degree of variety mixing (Fig. 4b). None of the studied varieties differed when comparing how much the beetles fed on a specific variety compared with if it was grown

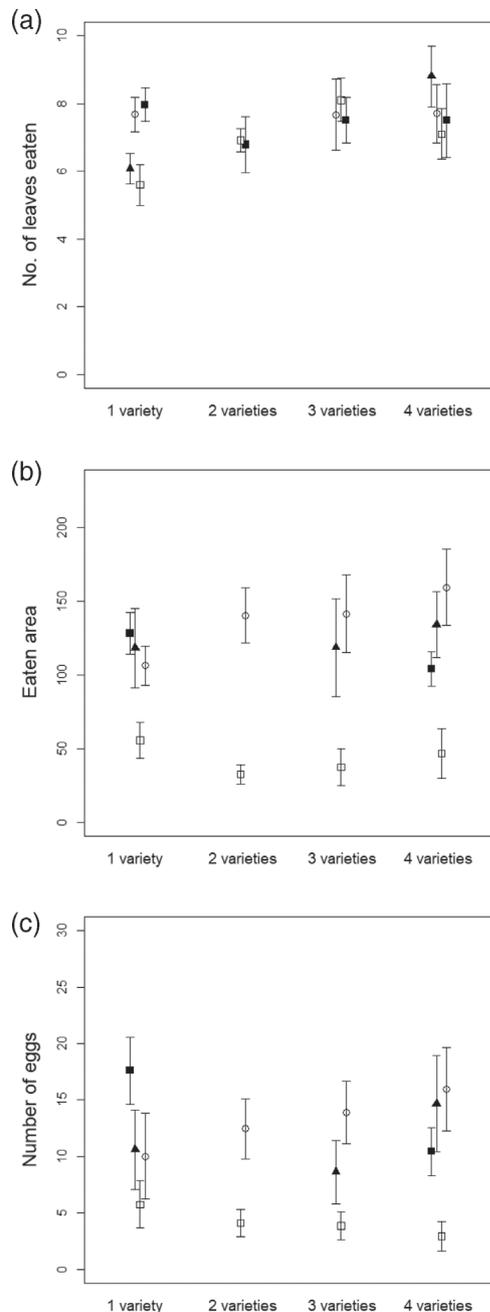


Figure 4 (a) Number of eaten leaves (mean \pm SE) out of 15 randomly selected per plant in a group of four willow plants potted individually and caged altogether with a perforated plastic. In each cage two adult leaf beetles (*Phratora vulgatissima*) were released for 4 weeks. Groups with one variety consist of either the variety 'Tora' ($n = 8$), 'Jorr' ($n = 8$), 'Loden' ($n = 4$) or 'Björn' ($n = 12$), groups of two varieties of 'Tora' and 'Loden' together ($n = 10$), three varieties of 'Tora', 'Jorr' and 'Loden' together ($n = 12$), and four varieties of all four genotypes together ($n = 12$). In (b) eaten area in $\text{mm}^2/2$ days (mean \pm SE) and in (c) number of eggs (mean \pm SE) laid on the different varieties by *P. vulgatissima*. The varieties were either planted as single varieties, two varieties together ('Tora' and 'Loden'), three varieties together ('Tora', 'Jorr' and 'Loden') or all four varieties together. All combinations were replicated 12 times, in total 84 containers. Symbols refer to 'Björn' (filled square), 'Jorr' (filled triangle), 'Loden' (open square) and 'Tora' (open circle).

in a single-variety or a multiple-variety group ('Tora': $F = 0.94$, $\text{denDF} = 26$, $P = 0.44$; 'Björn': $F = 1.28$, $\text{denDF} = 13$, $P = 0.28$; 'Jorr': $F = 0.20$, $\text{denDF} = 14$, $P = 0.82$; 'Loden': $F = 0.74$, $\text{denDF} = 25$, $P = 0.54$).

Egg-laying correlated well with feeding. More eggs were laid on leaves from the variety upon which the beetles had fed more (Pearson's correlation coefficient = 0.72, t -value = 8.67, $\text{d.f.} = 71$, $P < 0.001$). Overall, beetles laid fewer eggs on 'Loden' ($F = 7.3$, $\text{d.f.} = 3$, $P < 0.001$) (Fig. 4c). There were also less eggs on 'Björn' when grown in four-variety groups as compared with the single-variety group (t -value = -1.8 , $\text{d.f.} = 87$, P value = 0.08).

Only variety identity had a significant effect on larval survival (LR $\chi^2 = 9.9$, $\text{d.f.} = 102$, $P = 0.02$) and development (LR $\chi^2 = 47.0$, $\text{d.f.} = 96$, $P < 0.001$). Survival was lower on 'Loden' compared with the other varieties (percentage survival \pm SE; 'Björn': 76.0 ± 5.8 , 'Jorr': 80.0 ± 5.0 , 'Loden': 65.0 ± 5.2 , and 'Tora': 83.2 ± 4.3). Also, larval development was slower on 'Loden' than on the other varieties (mean number of days \pm SE; 'Björn': 14.2 ± 0.7 , 'Jorr': 14.9 ± 0.5 , 'Loden': 19.4 ± 0.9 , and 'Tora': 13.1 ± 0.5).

Discussion

Our predictions that willow plot diversity affects (positively or negatively) herbivore damage were not consistently supported over the 3 years of field studies or for the six types of herbivores investigated. We found less herbivore damage in mixed plots than in single-variety plots for the two most prevalent herbivore types in 2016, and although there were differences in mean numbers in the same direction, these were not statistically significant for the other years of the field experiments. We found no support for the expectation that the interaction of willow variety and the composition of the plots affected herbivory in any year. Herbivore damage levels among varieties grown in pure cultures varied only in the 2017 field study where 'Jorr' (*S. viminialis*) had lower levels of leaf chewers damage than the other three willow varieties. Levels of damage by the two sibling hybrid varieties 'Björn' and 'Tora' (*S. schwerinii* \times *S. viminialis*) were not significantly different from each other, and the variety 'Loden' (*S. dasyclados*) had higher levels than 'Tora' but not 'Björn'. Although this was not consistent for all 3 years of the study, the results indicate that, in some conditions, specific willow variety could play an important role in the level of damage by some herbivores. Our data do not allow us to make generalizations about the importance of phylogenetic relatedness and levels of damage by herbivores, but it is interesting that the impact of willow variety shifted over time. It may be that insect specialization is a part of the explanation (Castagneyrol *et al.*, 2014) and that leaf chewers are less narrow in their diet breadth. Earlier studies showed, for example, that *S. dasyclados* (e.g. 'Loden' in our study) is resistant to the more specialized gall midges (*Dasineura marginemtorquens*) and that *S. viminialis* ('Jorr' in our study) is its primary host plant (Glynn & Larsson, 1994; Rönnberg-Wastljung *et al.*, 2006). Even though the levels of gall midge damage were too low in our study to investigate patterns of herbivory, we surmise that the level of specialization between the host plant and herbivore is an important factor that needs to be accounted for in studies of stand heterogeneity and herbivore damage.

As such, the results from our field studies force us to reconsider the generality of our original expectations about bottom-up effects on herbivory. We discovered no general patterns indicating that specific varieties, variety mixes, or levels of stand diversity or composition consistently decreased herbivory damage. Instead, it may be that plant trait plasticity was higher than expected, that is, the plant traits in our willow varieties were more variable between years than we had assumed (Barton *et al.*, 2015). In our greenhouse experiments investigating associational effects on *P. vulgatissima*, we found that leaf beetle feeding choice and egg-laying were influenced only to some extent by plant diversity, further indicating weak bottom-up effects. Our results highlight the context-dependency of how plant stand heterogeneity affects the levels of damage by herbivores. Factors such as population levels of various types of herbivores, levels of host specificity of the herbivores, the combinations of various individual plant varieties in the plots, the plant physiological status (i.e. stage of willow growth in relation to root age and shoot growth after coppicing), and the levels of predators in the plots are likely to influence the levels of herbivory.

Our results indicate that in our system, heterogeneity facilitates increased predation. Predation levels were predicted to be higher in multiple-variety field plots as compared with single-variety plots. This is supported by our results from the field study of egg predation as well as by some cases from our plasticine caterpillar studies. We recorded up to five-fold variations in mean numbers of leaves damaged by some of the six herbivore types over 3 years in our study (Table 1) and we suggest that the patterns of herbivory and indicators of predation that we found in our field studies may be explained by the densities of the consumers. It is likely that the foraging behaviours of both the herbivores and the predators are influenced by inter- and intraspecific competition (Underwood, 2010; Parent *et al.*, 2014; Carrasco *et al.*, 2015). We did not measure insect densities in our study, but in those that have, herbivore number was not always related to herbivore damage (Koricheva & Hayes, 2018). Herbivore damage is the result of trophic interactions, and consequently our work supports Fernandez-Conradi *et al.* (2017) in suggesting that plant genotypic effects on herbivory are density-dependent.

Levels of predation and herbivory are tightly linked in the field, and this may explain why we found fluctuating prevalence of various types of predators and herbivores among the years. Disturbances caused by management practices, for example, coppicing of willows, are known to interfere with insect population dynamics and hence the densities of both herbivores and interlinked species such as predators (Björkman *et al.*, 2004; Thorbek & Bilde, 2004). We found relatively more bite marks in plasticine caterpillars placed in the multiple-variety plots in the year the willows were un-coppiced. The opposite occurred the year after it had been coppiced, in accordance with other studies showing that predator communities collapse after coppicing (Björkman *et al.*, 2004). Predation on leaf beetle eggs, however, revealed the same year higher predation in the multiple-variety plots. Our results indicate that this altered strength within trophic interactions plays a role for how plant heterogeneity affects herbivory. Mixing varieties and species as a strategy for pest control may be more suitable for more long-term cropping systems with less disturbance, and hence the management regime might be an important factor to take into account when evaluating the

potential role of mixed crops in, for example, integrated pest management.

We also found that predation seemed to be more dependent on specific varieties and specific mixtures than to an increase in plant heterogeneity *per se*. Our findings are in accordance with a growing number of studies that indicate variety identity and the specific mixture of varieties in a plot to be more important factors affecting various ecological processes than variety diversity in itself (Smithson & Lenne, 1996; Crawford & Rudgers, 2013; Hoeber *et al.*, 2018; Weih *et al.*, 2021). Egg predation was, for example, only higher in mixes including 'Björn' compared with pure cultures of 'Björn'. On plasticine caterpillars, there was more predation on 'Tora' when this variety was mixed with 'Jorr' than when it was grown in pure culture. This could be examples of the resource dilution effect (Otway *et al.*, 2005), mediated through herbivores. It could be either that certain varieties are more attractive and when the number of available varieties decreases as heterogeneity increases, herbivores are more abundant on them, or that certain varieties in a mixture are less attractive and herbivores are then more concentrated on the other varieties. Different prey types appear patchily in nature and their predators have likely adopted search strategies accordingly; perhaps, it is therefore not surprising that if herbivores can be affected by the resource dilution effect, their predators should follow (Russel, 1989).

To address our premise that direct associational effects acting on insect behaviour would affect levels of herbivore damage, we looked at *P. vulgatissima* feeding preferences to see if they choose to feed less on plants grown in multiple-variety plots compared with single-variety plots. In controlled experiments, we tested if associate effects, that is, the influence of neighbouring plants on a focal plant (Mervin *et al.*, 2017), could influence insect feeding choice. We found that some varieties were more attractive when grown in mixed cultures. The greenhouse experiment with willows arranged in single-variety groups showed that 'Tora' and 'Jorr' had higher levels of damage as compared with 'Loden' and 'Björn'. Even though 'Tora' and 'Björn' are full siblings, the leaf beetles prefer 'Tora', which could be an effect of variety differences in leaf chemistry (Hoeber *et al.*, 2020). Even though 'Loden' and 'Björn' were less preferred, feeding on them increased when they were grouped together with the other variety. Associational susceptibility, that is, when plants experience increased herbivory when growing together with preferred host plants (Agrawal, 2004), may explain these results. A mixed diet, that is, a large range of different food resources, may be a strategy to be able to digest the food (e.g. those high in secondary metabolites) more easily (Freeland & Janzen, 1974; Moritz *et al.*, 2018). It is also likely that above-ground competition affected plant traits and thus the choice of feeding sites in a way that was not measured in this experiment (Barbosa *et al.*, 2009). For example, it was shown that adding individual species or varieties to a mixed community significantly affected some community-level plant traits of the willows investigated here (Weih *et al.*, 2021), which also could have influenced the feeding preferences of herbivores.

We predicted that associational effects indirectly would affect the leaf beetles due to changes in food quality. As evidence, we expected to find that adult feeding, egg-laying and larval performance would differ depending on whether a variety was

grown in pure cultures or in a mix of varieties. In our no-choice feeding experiments, we found no effects of plant combination on adult preference in terms of feeding. Different life stages of an insect may respond differently to increased plant heterogeneity, which is why we performed tests on both adults and larvae. Larval survival and development were affected by willow variety, but we found no effect of willow variety composition. Either there were no strong complimentary effects such as altered plant traits within varieties among mixtures, or leaf beetles were not responsive to the effects.

As expected, the results from the controlled greenhouse study showed a strong correlation of the number of eggs laid with the amount of adult feeding of detached leaves. One exception can be noted: Egg-laying on the variety 'Björn' depended on variety composition of the willow community. Since the number of eggs is strongly related to the potential fitness of *P. vulgarissima* (Dalin, 2006), preferences in egg-laying sites may exert influences on beetle population dynamics and affect plant damage levels in the short as well as the long run.

Conclusions and future perspectives

Perceptions have historically varied on if plant heterogeneity has a stabilizing effect on higher trophic levels (McCann, 2000). Recent results in support of this stabilizing effect are from a meta-analysis by Reiss and Drinkwater (2018) that revealed higher yields in crop cultivar mixtures than monocultures, and that the diversity effects were stronger under pressure of disease and abiotic stressors. Higher genotypic diversity is argued to increase arthropod species and abundances, making systems that are more diverse more stable. Yet mechanisms to explain this and how plant communities best use available resources are poorly understood, especially in woody plant systems. The results from our work indicate that top-down forces influence the levels of damage caused by insect herbivores. Still, our results regarding plant variety diversity, identities of the plant community components and the effects on herbivory, predation and associated plant assemblages indicate strong temporal variation (variation with year since coppicing, which differentially could affect host plant traits, herbivore and predator communities) as well as strong dependencies on the species of herbivore studied. This indicates that factors other than plant species richness and species abundances are important. Future studies that focus on processes and mechanisms involved in trophic interactions will allow a better understanding of the factors that contribute to stabilizing pest populations in a longer perspective.

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Author contribution

IK, MW and CG designed the experiments; IK and CG performed the experiments; IK and CG analysed the data; IK and CG wrote the manuscript with significant input from MW.

Data availability statement

Data openly available in a public repository that issues datasets with DOIs.

Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Doc. S1. Supporting information.

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