Effects of plant diversity on the concentration of secondary plant metabolites and the density of arthropods on focal plants in the field

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Summary

1. The diversity of the surrounding plant community can directly affect the abundance of insects on a focal plant as well as the size and quality of that focal plant. However, to what extent the effects of plant diversity on the arthropod community on a focal plant are mediated by host plant quality or by the diversity of the surrounding plants remains unresolved.

2. In the field, we sampled arthropod communities on focal *Jacobaea vulgaris* plants growing in experimental plant communities that were maintained at different levels of diversity (one, two, four or nine species) for 3 years. Focal plants were also planted in plots without surrounding vegetation. We recorded the structural characteristics of each of the surrounding plant communities as well as the growth, and primary and secondary chemistry (pyrrolizidine alkaloids, PAs) of the focal plants to disentangle the potential mechanisms causing the diversity effects.

3. Two years after planting, the abundance of arthropods on focal plants that were still in the vegetative stage decreased with increasing plant diversity, while the abundance of arthropods on reproductive focal plants was not significantly affected by the diversity of the neighbouring community. The size of both vegetative and reproductive focal plants was not significantly affected by the diversity of the neighbouring community, but the levels of PAs and the foliar N concentration of vegetative focal plants decreased with increasing plant diversity. Structural equation modelling revealed that the effects of plant diversity on the arthropod communities on focal plants were not mediated by changes in plant quality.

4. *Synthesis.* Plant quality can greatly influence insect preference and performance. However, under natural conditions, the effects of the neighbouring plant community can overrule the plant quality effects of individual plants growing in those communities on the abundance of insects associated to this plant.

Key-words: biodiversity, insect community, *Jacobaea vulgaris*, phytochemistry, plant quality, plant species richness

Introduction

In plant communities, the presence and identity of neighbouring plants can greatly influence host plant location and host selection of insect herbivores. These effects are called associational effects (reviewed in Barbosa *et al.* 2009). Neighbouring plants can also influence characteristics of a focal plant, such as plant size and quality (primary and secondary chemistry; Barton & Bowers 2006; Temperton *et al.* 2007; Broz *et al.* 2010). These effects may result from competition between the focal plant and its neighbours that alters the availability of nutrients, light and space (Crawley 1997). In turn, changes in plant nutritional quality can greatly influence the interaction between plants and their multitrophic insect communities (Awmack & Leather 2002). However, whether the effects of neighbouring plants on the insect communities associated to a focal plant are mediated by the changes in focal plant quality or by the neighbouring community itself remains unresolved.

Apart from the identity of neighbouring plants, the diversity of the neighbouring plant community can also be an

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important factor that influences interactions between a focal plant and its insect community (Bezemer *et al.* 2004; Scherber *et al.* 2006; Unsicker *et al.* 2006; Kostenko *et al.* 2012; Wäschke *et al.* 2015). Specialist herbivore loads on a focal plant may be negatively related with plant diversity (*associational resistance hypothesis*, Tahvanainen & Root 1972). In contrast, the abundance of generalist herbivores on a focal plant (*associational susceptibility hypothesis*, Atsatt & O'Dowd 1976), as well as the abundance and diversity of the natural enemies of the herbivores, such as parasitoids (*enemies hypothesis*, Root 1973), is predicted to be higher in more diverse plant communities. Several factors may contribute to these effects of plant diversity, including the structure or height of the neighbouring vegetation, that directly affects the apparency of the focal plant as well as the size and composition of the local pool of insects that could subsequently 'spill over' to the focal plant (Kareiva 1983; White & Whitham 2000; Castagneyrol *et al.* 2013; Moreira *et al.* 2016).

Many studies have shown that host plant quality, characterised by the concentration of primary and secondary compounds in the plant, is an essential factor influencing preference and performance of herbivorous insects (reviewed in Awmack & Leather 2002). The vast majority of those studies have been performed in controlled environments and the role of plant quality in influencing above-ground insects in natural communities is less well understood. However, monospecific field experiments, for example with *Brassica oleracea* cultivars that differ in nutritional and chemical quality, have shown that the composition of the herbivore and predator community associated with a plant is significantly affected by the intraspecific variation in plant quality (Bukovinszky *et al.* 2008; Poelman *et al.* 2009). To what extend intraspecific variation in plant quality determines the composition of insect herbivore and predator communities in natural and hence diverse plant communities is an open question.

In the field, the nutritional quality of a focal plant can be influenced by the diversity of the neighbouring plant community. Recently, it was shown, for example that plant diversity can affect the expression of secondary metabolites in focal plants (Mraja *et al.* 2011; Wäschke *et al.* 2015). Three ecological theories predict that the concentration of secondary metabolites in a plant can be influenced by the diversity of the plant community. The *growth-defence trade-off hypothesis* (Herms & Mattson 1992) states that plants will allocate more resources to defence in more diverse plant communities as increased plant diversity will lead to increased competition for nutrients, water and light and hence to reduced growth of focal plants (Eisenhauer *et al.* 2009). In contrast, the *specialist–generalist dilemma hypothesis* (Van der Meijden 1996) states that the concentration of secondary plant compounds in a plant is expected to depend on the ratio of generalist and specialist herbivores in the community, whereby specialists prefer plants with high concentrations of defence compounds while generalists favour low defended plants. *The resource concentration hypothesis* (Root 1973) predicts that with increasing plant diversity, herbivore communities will change

from specialist to generalist dominated, as specialists prefer monospecific communities of their host plants. Hence, to withstand herbivory, the concentration of plant defence compounds in a focal plant should increase with increasing plant diversity.

A recent meta-analysis showed that levels of secondary plant chemicals generally increase during the ontogenetic development of a plant (Barton & Koricheva 2010). Plants in their reproductive stage are more important for plant fitness than vegetative plants and this may explain why they are better defended (Rhoades 1979; Agrawal 2004; Lankau & Kliebenstein 2009; Barton & Koricheva 2010). Moreover, reproductive plants are generally more apparent to insects due to the increased size and the presence of inflorescences, and more attractive due to the provision of nectar or other available resources (Rhoades & Cates 1976; Feeny 1976). Induced plant defence theory (Karban & Baldwin 1997) predicts that the increased exposure of flowering plants to insect herbivores will lead to increased levels of plant defence compounds, and this may also explain why reproductive plants will express higher levels of secondary plant compounds.

In a biodiversity field experiment, we examined how the diversity of the neighbouring plant community influences the nutritional quality and above-ground arthropod communities associated to focal *Jacobaea vulgaris* plants that were planted in the experimental plant communities. We further studied to what extent the arthropod communities on these focal plants are determined by the characteristics of the host plant and of the neighbouring plant community. *Jacobaea vulgaris* is a biennial or short-lived perennial monocarpic plant of the family Asteraceae. In the first year, a rosette of leaves is formed and flowering stems are produced in the second year. However, flowering may be delayed to later years when the plant has been damaged or when the size of the rosette is too small (Harper & Wood 1957; Van der Meijden & Van der Waals-Kooi 1979). The flowering stems can be more than 1 m tall and are highly apparent due to the bright yellow inflorescences (Kostenko & Bezemer 2013). *Jacobaea vulgaris* produces pyrrolizidine alkaloids (PAs), a well-studied group of nitrogen-containing secondary compounds that are toxic to a wide range of generalist insects, micro-organisms, mammals and humans (reviewed in Boppre 2011; Macel 2011). Specialist insects, in contrast, are not deterred by PAs but utilise them to locate hosts or sequester PAs for their own defence (e.g., Narberhaus *et al.* 2004). *Jacobaea vulgaris* harbours a rich insect fauna of more than 70 recorded species of herbivores (Harper & Wood 1957). Several studies have shown that there is a positive relationship between the concentration of PAs and plant size in this plant species (Hol, Vrieling & Van Veen 2003; Schaffner, Vrieling & van der Meijden 2003; Kostenko, Mulder & Bezemer 2013).

We hypothesised: (i) that an increase in plant diversity will result in increased competition (for space and available soil resources), impair plant survival, development and growth, and that this will lead to a decrease in concentrations of PAs in focal plants; and (ii) an increase in plant diversity will negatively affect the number of arthropods on vegetative focal

plants but will not affect the abundance of arthropods on reproductive focal plants. The reproductive plants are taller than most of the plants in the surrounding community independent of the diversity of that community; therefore, they will be highly apparent to insects in all experimental communities. Vegetative plants, in contrast, are concealed by the neighbouring plant community and this can directly hinder insects from finding their host plant.

In order to test these hypotheses, we set up a field experiment with plots in which we maintained plant communities at one, two, four and nine species diversity levels, and had plots without surrounding vegetation ('bare plots'). In each plot, we planted focal *J. vulgaris* plants in a fixed design and determined the growth and primary and secondary chemistry of vegetative and reproductive plants. We also recorded a number of characteristics of each plant community. Finally, we used a structural equation model to assess the strength and direction of alternative causal pathways linking the diversity of the neighbouring plant community to the abundance of arthropods on the focal plants. We hypothesised that (iii) the effect of plant diversity on the abundance of arthropods associated to the focal plants is mediated by changes in the chemistry of the focal *J. vulgaris* plants.

Materials and methods

EXPERIMENTAL DESIGN

A detailed description of the field experiment is presented in Kostenko *et al.* (2012). Briefly, in the summer of 2008, 70 plots (3 × 3 m), separated by paths (1 m wide) were established in an area of 25 m × 50 m within the nature restoration site Mossel (Ede, The Netherlands) on former arable land. The restoration site was 180 ha and agricultural practices were ceased in 1995. The plant diversity in the restoration grassland is around 12–15 species per 3 × 3 m^2 (TM Bezemer, personal observation). In September 2008, the vegetation was removed from each plot and the soil was tilled with a rotavator. Plots were sown with a single plant species (monocultures) or with mixtures of two, four or nine species randomly chosen from a pool of 12 local grassland species that naturally co-occur (in high abundance) with *J. vulgaris* in the studied area (grasses: *Anthoxanthum odoratum* L., *Agrostis capillaris* L., *Festuca rubra* L., legumes: *Lotus corniculatus* L., *Trifolium arvense* L., *Trifolium repens* L., other forbs: *Achillea millefolium* L., *Hypochaeris radicata* L., *Leucanthemum vulgare* Lamk., *Plantago lanceolata* L., *Tanacetum vulgare* L., *Tripleurospermum maritimum* (L.) W.D.J. Koch). The focal species *J. vulgaris* was not sown. There were 12 different monocultures, nine two-species, 11 four-species, and three nine-species mixtures (12 + 9 + 11 + 3 = 35 different plant communities, Table S1, Supporting Information). Each plant community (monoculture or mixture) was replicated twice using a complete randomised design (35 × 2 = 70 plots). The monocultures of *T. arvense*, *T. maritimum*, *A. capillaris* and *A. odoratum* (in total 4 × 2 = 8 plots) were excluded from the experiment because of poor establishment, but these species were present in mixed communities. Four of these plots initially sown with a single species were kept free of all vegetation, and served as 'no surrounding vegetation' treatment to enable comparing *J. vulgaris* performance in plots with and without surrounding vegetation. The other four plots were not included in the analyses of the experiment so that the final experimental design

consists of 66 plots. Initial sowing density was 4000 seeds per m^2. The sown species composition was maintained by hand-weeding from the beginning of the growing season (late April) until the end of the growing season (late August) throughout the years 2009–2011. Paths between plots were regularly mown during the growing season, and the experimental plots were not mown. To exclude large vertebrate herbivores, the experimental site was fenced.

In August 2009, when the sown plant communities had established and the four bare plots had been weeded regularly, 25 *J. vulgaris* seedlings with at least two fully developed leaves were planted in a regular grid of 5 × 5 plants in the central 1·2 × 1·2 m square of each plot (in total 25 *J. vulgaris* plants × 66 plots = 1650 focal plants). The distance between the plants was 0·3 m. The resident plant community surrounding the *J. vulgaris* plants was not removed in order to test the effects of the surrounding community on the establishment of the seedlings. In plots without surrounding vegetation, no other plants than the 25 focal *J. vulgaris* were present. The *J. vulgaris* rosettes were grown from seeds collected from plants growing in the direct vicinity of the experimental site. After germination, individual seedlings were transplanted into seedling trays filled with sterilised potting compost. Before planting in the field, plants were grown for 3 weeks in a greenhouse (21/16 °C day/night, 16-h photoperiod) and watered three times per week. Natural daylight in the greenhouse was supplemented by 400 W metal halide lamps (1 lamp per 1·5 m^2).

FOCAL PLANT AND COMMUNITY SAMPLING

In August 2011, 2 years after *J. vulgaris* rosettes had been planted in the field, a total of 1324 (out of 1650 planted) focal plants were recovered in the experimental plots. We intended to collect four reproductive and four vegetative plants in each plot. However, only 424 of the *J. vulgaris* plants produced flowering stems (reproductive stage) 2 years after transplanting and flowering was not evenly distributed among the plots (see Results). Therefore, in 17 of the 66 plots, fewer than four (on average 2) and in 18 other plots (out of 66), no reproductive plants could be collected. The above-ground plant parts (rosettes of leaves or rosettes with flowering stems) were clipped-off and placed in a labelled paper bag. The fifth youngest fully expanded leaf from each rosette and flowering plant was removed with a razor blade, immediately frozen at −20 °C, freeze-dried, weighed and ground for chemical analysis. The remaining of each plant was oven-dried for 48 h at 70 °C, and total shoot plant dry weight was determined. At the end of August, plant community measurements were made in each plot, to estimate the structural complexity of the community. For each plant community, the percentage cover of plant species was recorded in two 1 m^2 quadrants along a diagonal transect within each plot. The total percentage cover can exceed 100% because plants in a community can overlap. The height of the vegetation was measured using the vertical drop disc method (Stewart, Bourn & Thomas 2001). The disc weighed 200 g, had a diameter of 300 mm, and was released from a 1·5-m height. The height was measured at 10 random locations within each plot. One week after plant sampling, soil cores of 15-cm depth and 2·5-cm diameter were collected from each experimental plot at five random positions. The soil samples were pooled per plot and used for chemical analysis.

ARTHROPOD ABUNDANCE

Arthropods at all stages of their development (eggs, immature and adults) on each *J. vulgaris* plant were collected on four occasions

from May to August 2011. During each collection, all plants were carefully inspected between 10:00 h and 16:00 h and all arthropods that were observed on a plant were collected using an aspirator by three collectors distributed evenly over the field. Each collector inspected all 1324 plants, spending an approximately equal amount of time at each plant at all diversity levels. All arthropods were stored individually in 70% ethanol in labelled Eppendorf tubes. Most arthropods were identified to species or family level (see Table S4). All arthropod species were assigned to feeding group (specialist herbivore, generalist herbivore, predator, pollinator, detritivore and omnivore) based on their feeding strategy and the degree of specialisation. We focused our further analyses on three major groups of arthropods: specialist herbivores of *J. vulgaris*, generalist herbivores and carnivores (predators and parasitoids). The number of arthropods on individual plants collected at each date was relatively low (1st collection – 0·4; 2nd – 2; 3rd – 2; and 4th – 0·1 arthropods per plant) and data from the four collections were therefore pooled for each plant. We also calculated occurrences of arthropods as the proportion of plants with arthropods (independent of their density) within each plot to take into account that some arthropods occur in aggregated fashion (Figure S3).

CHEMICAL ANALYSES

For chemical analyses, we randomly selected four vegetative and four reproductive focal *J. vulgaris* plants per plot. However, in some plots, there were fewer than four vegetative or reproductive plants available resulting in total 259 vegetative and 157 flowering plants that were subjected to the chemical analyses. Leaf carbon (C) and nitrogen (N) concentrations were determined using a Flash EA1112 CN analyser (Interscience, Breda, The Netherlands). PA analysis of leaf and root samples was carried out using liquid chromatography-tandem mass spectrometry (LC-MS/MS) following the procedure outlined in Kostenko, Mulder & Bezemer (2013). In brief, 5 mg of freeze-dried ground plant material was extracted with 0·5 mL 2% formic acid solution containing heliotrine (1 µg mL^{-1}) as internal standard. After centrifugation and filtration, 25 µL of the extracted filtrate was diluted with 975 µL of 10 mM ammonium hydroxide solution and 10 µL was injected in a Waters Acquity ultra-performance chromatographic system coupled to a Waters Quattro Premier tandem mass spectrometer (Waters, Milford, MA, USA). Separation and mass spectrometric detection of the PAs was as described in Cheng *et al.* (2011) and Appendix S2. Data were processed using Masslynx 4.1 software. Mineral N content (NH_4^+ and NO_3^-) in soil samples was determined colorimetrically in the $CaCl_2$ extraction using a Traacs 800 autoanalyser (TechniCon Systems Inc, Oakland, CA, USA). The C:N ratio in soil samples was measured on a FlashEA 1112 Series NC soil analyser (Thermo Scientific, Waltham, MA, USA). pH was measured in 2:5 dry soil : water suspensions. The percentage organic C was determined according to Nelson & Sommers (1982) and available P according to Olsen *et al.* (1954) and measured at 720 nm (Table 1).

DATA ANALYSES

To fulfil the requirements of normality and homogeneity of variances, data were log- or square-root transformed. Proportions data were arcsine square-root transformed. To examine the effect of plant diversity on the arthropod abundance, focal plant growth and chemistry, we used mixed-effects models with plant diversity (0–9 species and 1–9 species) as continuous fixed factor to incorporate the continuity of plant diversity in the analysis. In these analyses, plot identity was

included as random factor to incorporate that multiple plants were sampled in each plot. Plant diversity was included as fixed factor as it was manipulated treatment in our experiment. The models for the vegetative and reproductive plants were run separately because of uneven distribution of flowering plants among the plots. We also examined whether the proportion of plants with arthropods per plot was affected by the diversity of the neighbouring community using a general linear model. The results of these analyses are presented in Figure S3. To test whether the number of reproductive plants in the community affected the abundance of arthropods on vegetative plants, we used a Pearson's product-moment correlation. As only a subset of the focal plants was subjected to chemical analyses, we first performed analyses of the arthropod abundance on the full data set including all plants, and then repeated all analyses using the smaller subset of the data. The results of the analyses of the subset of the data are presented in Table S5. The effects of plant diversity on the vegetation and soil characteristics were analysed using general linear models with plant diversity (0–9 species and 1–9 species) as continuous loglinear fixed factor. To test the effects of proportion of legumes, grasses or other forbs in the vegetation on *J. vulgaris* biomass and chemistry (N and PA concentrations), general linear models were used (Table S7). The correlation between PA and N concentrations was tested using a Pearson's product-moment correlation. Data were analysed using R statistical language, version 3.0.1 (R Development Core Team 2014).

STRUCTURAL EQUATION MODELLING

We used structural equation modelling (SEM) procedures (Grace 2006) to explore the strength and direction of pathways linking plant diversity and arthropod abundance on the focal *J. vulgaris* plants in biodiversity plots represented in the Fig. 1. As characteristics of the focal plants in our models we used plant shoot biomass, nitrogen and total PA concentration. The characteristics of the neighbouring vegetation included plant cover as a proxy of competition with surrounding plants for light and space, and height of the vegetation as a proxy of the community apparency. Plant diversity (1–9 plant species) was included as fixed continuous factor to incorporate the continuity of plant diversity in the analysis. The models for the vegetative and reproductive plants were run separately. We only examined models in which bare plots were excluded and to develop these models we used the subset of plants that were chemically analysed. All variables used in the SEM were observed variables. To improve the normality and stabilise variances, we transformed the data in the same way as in the univariate analyses. Structural equation modelling was carried out using the *lavaan* package in R. All final models provided good fit to the data (Table S3). Additional information about the SEM procedure is presented in Appendix S3.

Results

ARTHROPOD RESPONSES

The arthropod fauna associated to focal *J. vulgaris* plants was dominated by specialist herbivores, such as *Aphis jacobaeae* Schrank (Hemiptera: Aphididae), *Tyria jacobaeae* L. (Lepidoptera: Arctiidae) and *Longitarsus jacobaeae* Waterhouse (Coleoptera: Chrysomelidae) that accounted for 87% of the total number of collected arthropods (Table S4). The total number of arthropods on vegetative *J. vulgaris* plants decreased with increasing diversity of the neighbouring

Table 1. Vegetation and soil characteristics (mean ± SE) of experimental plots that were sown with one, two, four or nine species or kept without vegetation (0). Asterisks indicate significant effects based on a general linear model with plant diversity as fixed loglinear factor and bare plots included or excluded from the model. Asterisks indicate significant effect at ***$P < 0.001$; **$P < 0.01$; the brackets indicate marginally significant effect at $P < 0.06$; the absence of asterisks indicates that the effect is not significant

| Plant diversity	Total plant cover (%)	Vegetation height (cm)	pH	C:N ratio	Soil mineral N ($NH_4^+ + NO_3^-$) (mg kg^{-1})	P (mg kg^{-1})	Organic matter (%)
0	0 ± 0	0 ± 0	5·13 ± 0·05	16·4 ± 0·23	1·86 ± 1·02	118·7 ± 8·3	3·77 ± 0·15
1	132 ± 7·0	10·2 ± 1·4	5·10 ± 0·02	16·7 ± 0·13	1·91 ± 0·37	117·2 ± 2·1	3·97 ± 0·07
2	152 ± 7·0	8·8 ± 0·4	5·14 ± 0·01	16·6 ± 0·10	2·15 ± 0·47	115·4 ± 2·5	3·82 ± 0·06
4	160 ± 6·3	11·2 ± 0·4	5·17 ± 0·01	16·7 ± 0·08	3·95 ± 0·94	114·7 ± 1·9	3·97 ± 0·07
9	166 ± 8·2	13·3 ± 0·8	5·13 ± 0·04	16·8 ± 0·39	3·71 ± 1·66	114·5 ± 5·0	4·04 ± 0·09
Bare plots included	$F_{1,64} = 34·25$***	$F_{1,64} = 43·11$***	$F_{1,64} = 3·15$	$F_{1,64} = 1·36$	$F_{1,64} = 2·29$	$F_{1,64} = 0·92$	$F_{1,64} = 1·91$
Bare plots excluded	$F_{1,60} = 9·52$**	$F_{1,60} = 11·31$**	$F_{1,60} = (3·88)$	$F_{1,60} = 0·58$	$F_{1,60} = 1·82$	$F_{1,60} = 0·44$	$F_{1,60} = 0·85$
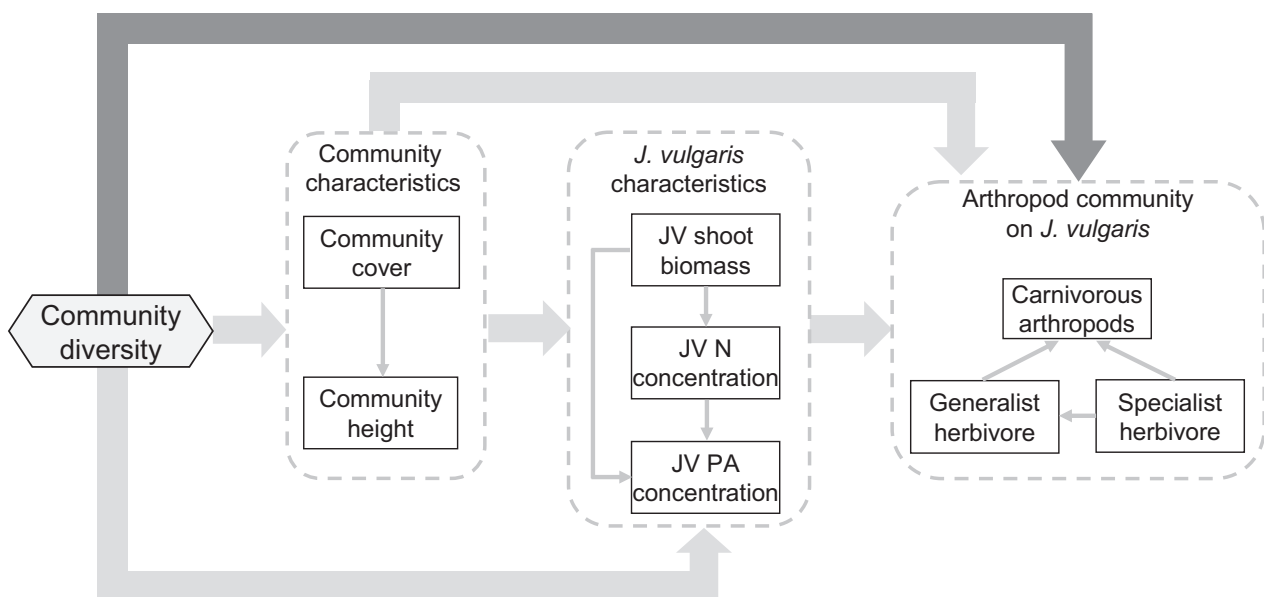
Fig. 1. Initial conceptual model describing the potential direct and indirect (mediated by the changes in the community characteristics or quality of focal *Jacobaea vulgaris* (JV) plants) effects of diversity of the neighbouring plant community on arthropod abundance associated to focal *J. vulgaris* plants. The hexagon around the 'Plant diversity' variable indicates the manipulated treatment and that this variable was included as fixed continuous factor in the models. The direct effect of community diversity on arthropod abundance is represented by dark grey arrow; the indirect effects of community diversity are represented by the light grey arrows.

community (0–9 species: $F_{1,64} = 6·0$, $P = 0·017$; 1–9 species: $F_{1,60} = 8·03$, $P = 0·0063$, Fig. 2). The abundance of specialist herbivores on vegetative plants also decreased with increasing plant diversity when bare plots were excluded from the model (0–9 species: $F_{1,64} = 2·80$, $P = 0·099$; specialists; 1–9 species: $F_{1,60} = 4·33$, $P = 0·042$, Fig. 2), whereas the abundance of generalist herbivores on vegetative plants was not significantly affected by plant diversity (0–9 species: $F_{1,64} = 1·08$, $P = 0·30$; 1–9 species: $F_{1,60} = 0·60$, $P = 0·44$, Fig. 2). The abundance of carnivorous arthropods associated to vegetative plants decreased with increasing diversity of the neighbouring community (0–9 species: $F_{1,64} = 5·05$, $P = 0·028$; 1–9 species: $F_{1,60} = 6·09$, $P = 0·017$, Fig. 2). There was no effect of plant diversity on the abundance of arthropods associated to reproductive *J. vulgaris* plants ($P > 0·05$ in all cases). The abundance of arthropods on

vegetative plants did not correlate with the number of the reproductive plants in the community ($P > 0·05$ in all cases).

PLANT COMMUNITY AND FOCAL PLANT CHARACTERISTICS

Total plant cover and height of the vegetation increased significantly with increasing plant diversity (Table 1). *J. vulgaris* survival ($F_{1,64} = 12·05$, $P = 0·0009$) and the number of flowering focal plants per plot decreased (0–9 species: $F_{1,64} = 5·84$, $P = 0·019$; 1–9 species: $F_{1,60} = 4·99$, $P = 0·029$) with increasing plant diversity. Plant diversity did not significantly affect plant biomass of vegetative (0–9 species; shoot: $F_{1,64} = 1·16$, $P = 0·29$; root: $F_{1,64} = 1·77$, $P = 0·19$, Fig. 3) and reproductive (0–9 species; shoot: $F_{1,46} = 0·37$, $P = 0·55$; root: $F_{1,46} = 1·31$, $P = 0·39$, Fig. 3)
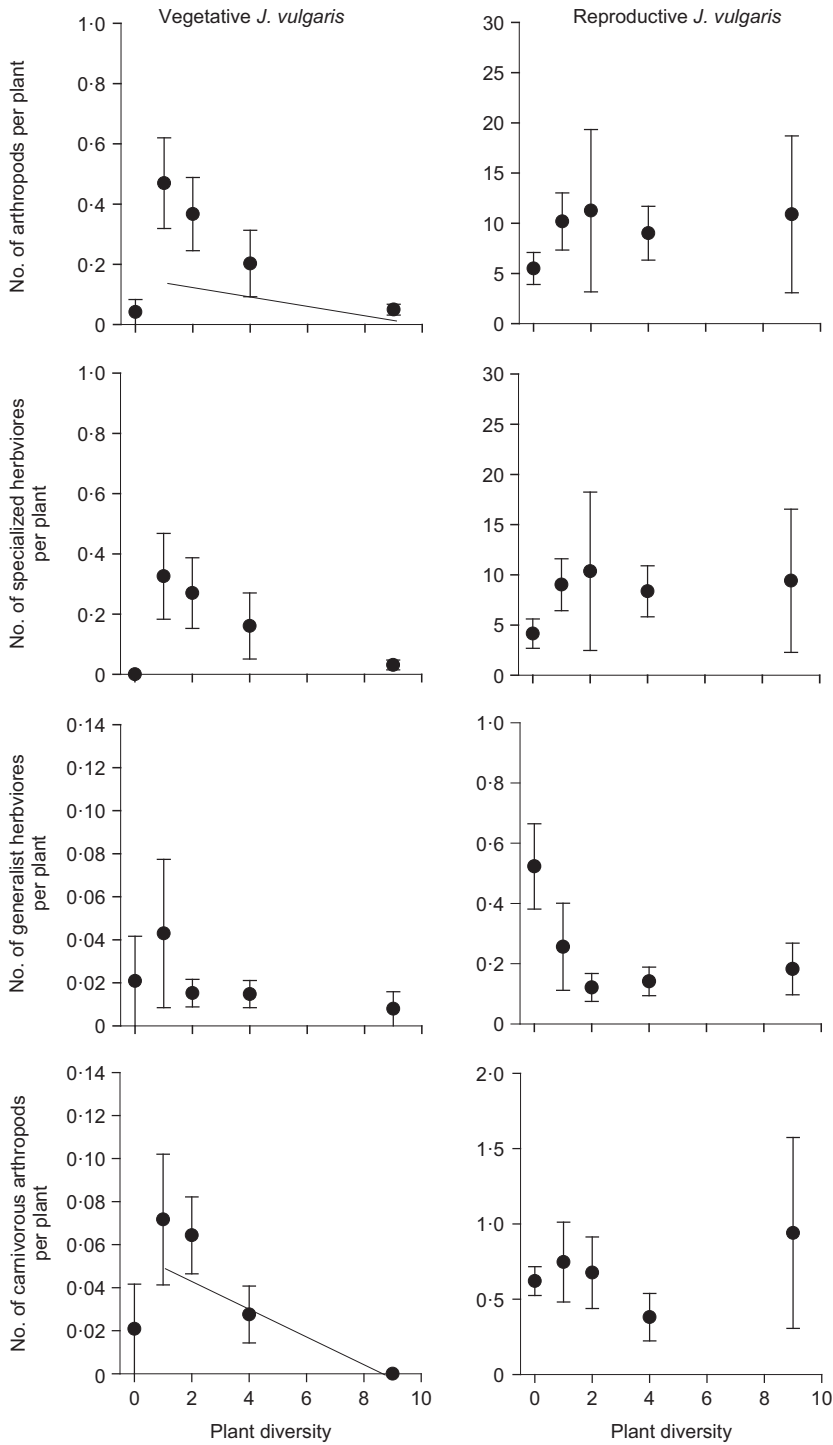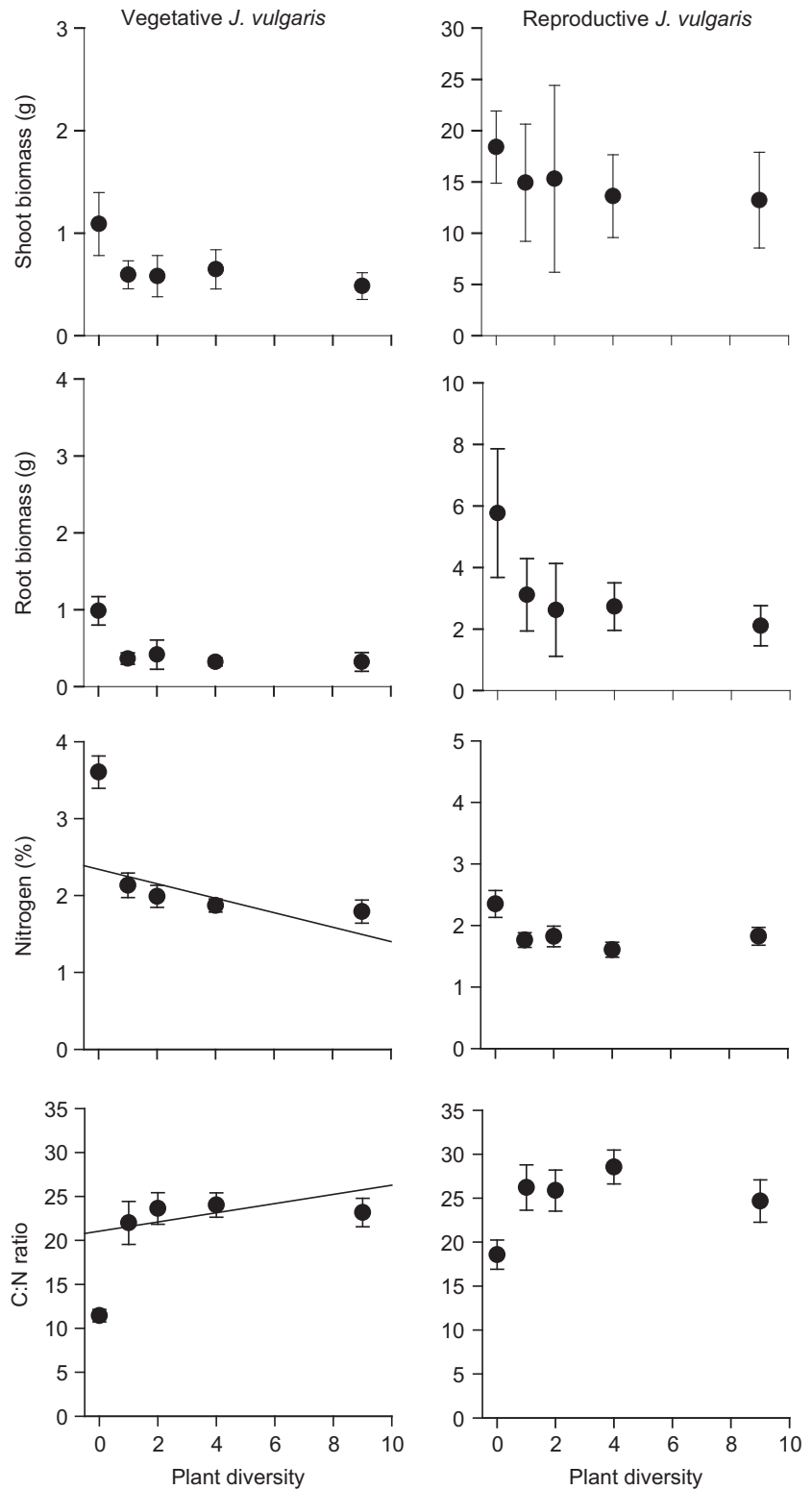
Fig. 2. Effect of diversity of the neighbouring community on total number of arthropods, specialist herbivore abundance, generalist herbivore abundance, and the abundance of carnivorous arthropods on the vegetative and reproductive focal *Jacobaea vulgaris* plants. Means are shown (calculated based on average values per plot ± between-plot SE). The average value per plot is calculated as total number of arthropod individuals in a plot divided by the number of vegetative or reproductive plants in the same plot. Lines indicate a significant relationship with plant diversity (0–9 species) based on the mixed-effects model.

focal plants. The leaf N concentration of vegetative focal plants decreased (0–9 species: $F_{1,64} = 16.41$, $P = 0.0001$, Fig. 3) and C:N ratio increased (0–9 species: $F_{1,64} = 10.60$, $P = 0.0018$, Fig. 3) with increasing plant diversity. The concentration of N (0–9 species: $F_{1,46} = 3.38$, $P = 0.072$, Fig. 3) and C:N ratio (0–9 species: $F_{1,46} = 3.34$, $P = 0.074$, Fig. 3) in leaves of reproductive focal plants was not significantly affected by plant diversity. When the analyses were limited to plots with surrounding vegetation (1–9 species), there was no effect of plant diversity on focal plant biomass, leaf N

concentration and C:N ratio for both vegetative and reproductive plants ($P > 0.05$ in all cases).

Overall, the PA concentration of the focal plants tended to decrease with increasing diversity of the neighbouring community (Fig. 4). The effect of plant diversity on the total PA concentration was statistically not significant (vegetative: $F_{1,64} = 3.04$, $P = 0.086$, reproductive: $F_{1,46} = 1.64$, $P = 0.21$, Fig. 4). However, a significant negative effect of plant diversity was observed for jacobine- and senecionine-type PAs in the leaves of vegetative focal plants (0–9 species:

Fig. 3. Effect of the diversity of the neighbouring community on the aboveground plant biomass, leaf N concentration and C:N ratio of the vegetative and reproductive focal *Jacobaea vulgaris* plants. Means ± between-plot SE are shown. Lines indicate a significant relationship with plant diversity (0–9 species) based on the mixed-effects model.

$F_{1,64} = 6.76$, $P = 0.012$; $F_{1,64} = 12.34$, $P = 0.0001$ respectively, Fig. 4). When bare plots were not included in the model, there was no significant effect of plant diversity on the total PA concentration or different types of PAs for both vegetative and reproductive focal plants ($P > 0.05$ in all cases) except on senecionine-type PAs in the leaves of vegetative focal plants ($F_{1,60} = 5.35$, $P = 0.024$). Total PA concentration positively correlated with N concentration for both vegetative (0–9 species: $r = 0.54$, $P < 0.0001$; 1–9 species: $r = 0.55$, $P < 0.0001$) and reproductive (0–9 species: $r = 0.34$, $P < 0.0001$; 1–9 species: $r = 0.40$, $P < 0.0001$) plants.
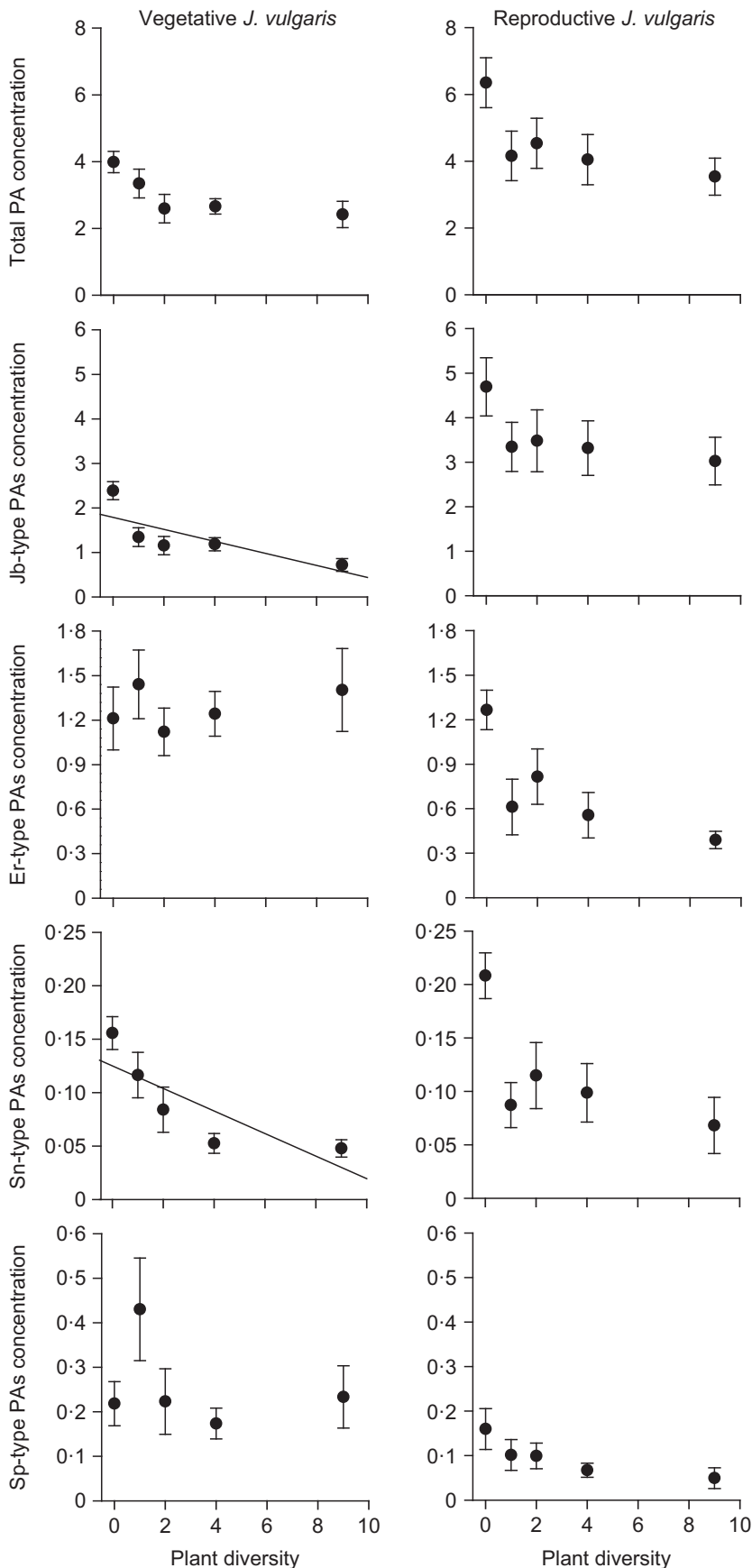
Fig. 4. Effect of diversity of the neighbouring community on the total PA concentration and the concentration of jacobine-type (Jb), erucifoline-type (Er), senecionine-type (Sn) and seneciphylline-type (Sp) PAs (mg g^{-1} dw) in the leaves of the vegetative and reproductive focal *Jacobaea vulgaris* plants. Means ± between-plot SE are shown. Lines indicate a significant relationship with plant diversity (0–9 species) based on the mixed-effects model.

SEM: DIRECT AND INDIRECT EFFECTS OF PLANT DIVERSITY

The final SEM for vegetative *J. vulgaris* plants explained 23%, 6% and 6% of the variation in the abundance of carnivorous arthropods, specialist herbivores and generalist herbivores (accordingly) associated to the focal *J. vulgaris* plants (Fig. 5, Table S3). There was a direct negative path from plant diversity to the abundance of carnivorous arthropods (Fig. 5). Plant diversity had also two indirect effects on the abundance of carnivorous arthropods. First, plant diversity enhanced the abundance of carnivorous arthropods by promoting the height of the surrounding community. Second, plant diversity decreased the abundance of carnivorous arthropods via increased vegetation cover in the

community that reduced the shoot biomass of *J. vulgaris* focal plants. However, the indirect pathways (the strength of the indirect effect = 0·08, $P = 0.099$ and -0.09, $P = 0.053$) were offsetting and less important in explaining the variation in carnivorous arthropod abundance than the direct pathway (-0.40). Interestingly, there were indirect negative links from plant diversity to N concentration in focal plants (-0.12, $P = 0.030$) through the increased percentage plant cover in a community, and to PA concentration in the focal plants (-0.076, $P = 0.034$) through the increased percentage plant cover in a community that in turn reduces the biomass of the *J. vulgaris* plants. The abundance of specialist was negatively and that of generalist herbivores was positively associated with PA concentration of *J. vulgaris* focal plants (Fig. 5). However, the
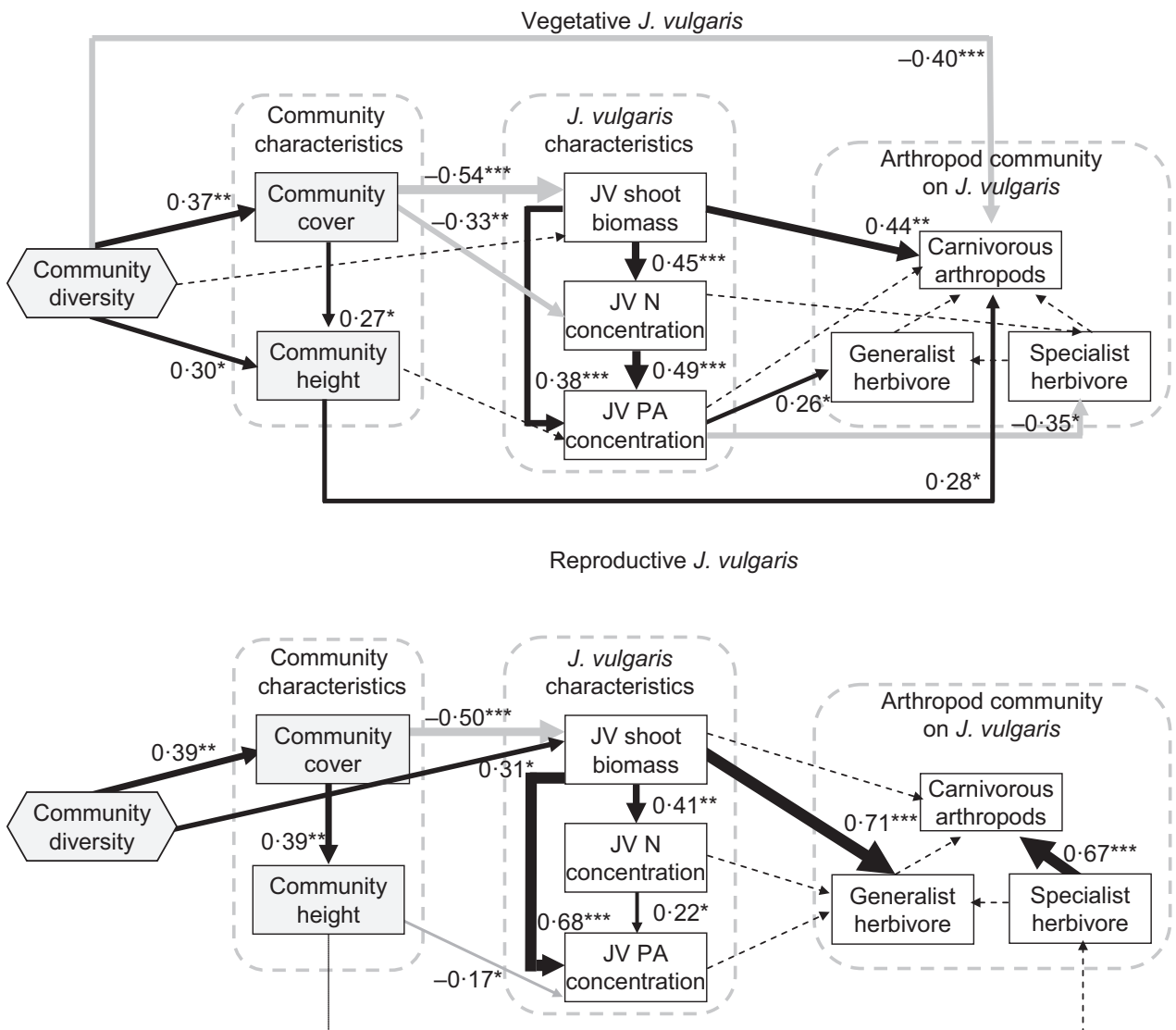
Fig. 5. Final structural equation models illustrating the strength and direction of the relationships among the characteristics of the neighbouring community, focal plant characteristics and arthropod abundance associated to the focal vegetative and reproductive *Jacobaea vulgaris* plants. Dark grey and light grey arrows denote positive and negative significant effects respectively. The dashed lines show non-significant effects at $P > 0.05$ that were retained in the final model. Arrow widths are proportional to standardised path coefficients that are shown next to the arrows and its significance is denoted as ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$. The strength of the direct paths corresponds to the path coefficient.

indirect effects of plant diversity on the abundance of herbivorous insects mediated by changes in plant PA concentration were not significant (specialists: 0·026, $P = 0·14$; generalists: $-0·019$, $P = 0·14$).

The final SEM for reproductive *J. vulgaris* plants explained 47% of the variation in the abundance of carnivorous arthropods, 36% of the variation in the abundance of generalist herbivores and 1% of the variation in the abundance of specialist herbivores associated to the focal *J. vulgaris* plants by indirect pathways (Fig. 5, Table S3). The abundance of generalist herbivores was positively associated to the biomass of the focal *J. vulgaris* plants. However, the indirect positive pathway between plant diversity and the abundance of generalist herbivores through *J. vulgaris* shoot biomass was not significant (0·22, $P = 0·063$). The abundance of carnivorous arthropods was strongly positively linked to the specialist herbivore abundance but was not significantly associated to any focal plant or community characteristics measured in our experiment (Fig. 5). There were several indirect pathways connecting plant diversity and PA concentration in reproductive plants, but only the negative path showing that plant diversity negatively affects PA concentration by promoting plant cover in the community and lowering the biomass of the focal plants, was statistically significant ($-0·13$, $P = 0·037$). Chemistry of reproductive focal plants (N or PA concentration) was not significantly associated with arthropod abundances (Fig. 5).

Discussion

Our study shows that, in the field, the composition and concentration of secondary plant compounds in a focal plant is influenced by the neighbouring plant community. The levels of almost all PA groups (i.e. Jb-type and Sn-type PAs) and the total PA concentration of vegetative *J. vulgaris* plants were lowest in the plots with the highest species diversity (nine plant species) and highest in the plots without surrounding vegetation. The abundance of arthropods found on these focal plants also decreased with increasing diversity of the neighbouring plant community. However, SEM revealed that the effects of plant diversity on arthropod abundances on vegetative *J. vulgaris* were not mediated by the effects of plant diversity on the chemistry of the focal plants. Below, we first discuss the effects of plant diversity on plant defence chemistry and subsequently the direct and indirect (via the focal plant) effects of plant diversity on above-ground arthropods on focal plants.

There are several possible explanations for the observed diversity effects on PA concentrations in focal *J. vulgaris* plants. Increasing plant diversity generally leads to an increase in the overall productivity of the plant community (reviewed in Gross *et al.* 2014). More productive plant communities are usually denser, which can lead to increased competition for space and light, as well as soil nutrient depletion (Spehn *et al.* 2000; Lorentzen *et al.* 2008; Eisenhauer *et al.* 2009; Oelmann *et al.* 2011). The focal plant in our study, *J. vulgaris*, is a poor competitor and increased competition in more diverse plant communities can lead to reduced growth

of the focal plants (McEvoy *et al.* 1993). SEM shows that plant diversity indirectly suppressed the growth of vegetative *J. vulgaris* plants by promoting the cover of the surrounding plant community (which we assume is a proxy for competitive effects) and thus reducing the number of open spaces on the ground that are essential for *J. vulgaris* rosette development (McEvoy *et al.* 1993). In addition, we found that forbs in the neighbouring community negatively affected the growth and the ability of focal plants (both vegetative and reproductive) to produce PAs. *Jacobaea vulgaris* is a forb species and possibly, in communities with high abundance of forbs, focal plants suffered from increased competition for available resources. Several studies have shown that total shoot PA concentration of *J. vulgaris* plants is positively related to the root biomass (Hol, Vrieling & Van Veen 2003; Schaffner, Vrieling & van der Meijden 2003). Furthermore in our study, SEM revealed a positive path between the total PA concentration and shoot biomass in both reproductive and vegetative plants. As the root biomass of *J. vulgaris* is highly positively correlated with shoot biomass in the field ($r = 0·96$; data not shown), we speculate that increased plant diversity led to increased competition for *J. vulgaris*, resulting in smaller plants, which, in turn, led to the observed decrease in PA concentrations. Importantly, when the effect of plant size was removed from the models, a significant effect of plant diversity on the PA concentration in focal plants remained (Table S6) suggesting that the diversity effects on plant defence chemistry are not fully mediated by diversity effects on plant size (see also Fig. 5).

Theory predicts that the production of plant defence compounds can be (partly) explained by the availability of resources in the soil (Bryant, Chapin & Klein 1983; Coley, Bryant & Chapin 1985; Herms & Mattson 1992). Some authors have argued that diverse plant communities may use limiting resources more effectively than simple communities (e.g., Oelmann *et al.* 2011). An increase in plant diversity could therefore lead to reduced N availability in the soil. However, even though the relationship was not statistically significant, in our study, the mineral N content in the soil increased with increasing plant diversity and was twice as high in high diverse plant communities as in monospecific communities, possibly due to an increase in legume abundance in more diverse plant communities (Table 1, Figure S4). The total PA concentration was not correlated with soil mineral N content (data not shown) and was weakly (vegetative plants) or not affected (reproductive plants) by the abundance of legumes in the neighbouring communities (Table S7). It is important to note, though, that N availability in the soil was only measured once at the end of the season, while plants had been growing for 2 years in the soil. Hence, leaf N concentration may be better indicator of nitrogen availability to the plant, and in our study foliar N concentration in focal plants decreased with increasing plant diversity. Foliar N concentration was positively correlated with total PA concentration for both vegetative and reproductive plants. However, SEM revealed a significant indirect pathway connecting plant diversity and PA concentration via changes in leaf

nitrogen concentration (the pathway that did not include plant size) only for vegetative plants. As this was only true for vegetative plants, it suggests that the correlation between plant N and PA concentration is stronger at the rosette stage when the plant has a low shoot to root ratio.

Previous studies suggested that plant diversity can also influence the concentration of plant defence compounds in focal plants via the effects of diversity on herbivory (Mraja *et al.* 2011; Wäschke *et al.* 2015). For example, Mraja *et al.* (2011) reported that increased concentration of catalpol, an induced defence compound in *Plantago lanceolata*, positively correlated with herbivore damage at increasing plant diversity in a grassland biodiversity experiment. In the present study, we did not estimate the amount of herbivore damage on the focal plants. However, similar to the previous study, PA concentration was positively related to the number of specialised herbivores colonising *J. vulgaris* rosettes. Although the diversity effect was opposite to the previous study as both the PA concentration and the number of specialised herbivores decreased with increasing plant diversity. This finding is in accordance with the prediction of the *specialist–generalist dilemma* hypothesis (Van der Meijden 1996). Therefore, it is possible that the differences in PA concentrations were directly (defence induction) related to differences in herbivore pressure on the focal plants in the different experimental diversity plots. However, it is important to note that previous experiments demonstrated that PA production in *J. vulgaris* is not induced in response to shoot herbivory (Hol *et al.* 2004). Thus, in our system, the diversity effects on plant chemistry are most likely not mediated by the differences in herbivore abundances.

Intraspecific variation in the expression of plant defence compounds can also have a genetic basis. The PA composition in *J. vulgaris* plants is partially genetically determined (Vrieling, De Vos & Van Wijk 1993; Macel, Vrieling & Klinkhamer 2004). We did not measure genetic variation among the focal plants. However, as all focal plants originated from seeds collected from one *J. vulgaris* population, we assume that the genetic variation among individual plants was relatively low. Finally, other characteristics of the neighbouring community, such as the identity and diversity of plant functional groups (Table S7), allelopathic effects or interspecific plant-soil feedback effects may also be responsible for changes in plant growth and PA concentrations of *J. vulgaris*. For example, in previous studies, it has been shown that other plant species can have a strong effect on *J. vulgaris* biomass and PA concentration via changes in the composition of the soil microbial community (Van de Voorde, Van der Putten & Bezemer 2011; Kos *et al.* 2015). Emission of volatile compounds by neighbouring plants may also influence the resistance of a focal plant by inducing the expression of defensive chemicals in the focal plant (reviewed in Heil & Karban 2010). However, this needs further investigation.

As far as we are aware, the question how plant diversity affects the levels of plant defence compounds has been addressed by few studies so far (Broz *et al.* 2010; Mraja *et al.* 2011; Wäschke *et al.* 2015). The strength and direction of plant diversity effects in those studies differed from those observed in our study, and also varied among the above-mentioned studies even when the same defence compounds were examined (i.e. iridoid glycosides; Mraja *et al.* 2011; Wäschke *et al.* 2015). Intraspecific variation in plant defence chemistry at small spatial scales can have important consequences for a wide variety of ecosystem processes, such as herbivory, disease dynamics, nutrient cycling and decomposition (Crawley 1997). Recently, it has also been shown that intraspecific variation in plant chemistry triggered by plant diversity (even in the short term) can persist in new generations (Hennion *et al.* 2016). Whether the differences in the PA profiles of focal *J. vulgaris* plants in our study will be inherited by the new generation is an interesting question that should be investigated in the future. Field studies that examine the plasticity of plant defences in relation to environmental factors and an understanding of biosynthetic pathways involved in the synthesis of particular defensive metabolites are necessary to understand and predict the effects of plant diversity on plant defence chemistry.

In a previous study, we reported the abundance of insects on the same focal plants in the experimental plots during the first year after transplantation when all *J. vulgaris* were still in the rosette stage (Kostenko *et al.* 2012). In the current study, we collected arthropods on vegetative and reproductive focal plants when plants had been growing for 2 years in the experimental plots. The results of the current study are in agreement with the previous study as we found in both years fewer specialised herbivorous insects on vegetative plants in the most diverse communities. This suggests that plant diversity provides associational resistance to individual plants growing in those communities. However, in contrast to results obtained during the first year of the experiment, in the second year very few arthropods were found on the vegetative focal plants growing in the bare plots without neighbouring vegetation. This may have been due to the low number of vegetative plants present in the bare plots as most plants in those plots were flowering during the second year, and because the rosettes are less apparent for insects than the flowering *J. vulgaris* plants. The difference in apparency may also explain the much lower number of arthropods that overall were found on vegetative compared to reproductive focal plants. However, the abundance of arthropods on vegetative *J. vulgaris* plants did not correlate with the number of the reproductive plants in a community. Finally, along with the differences in apparency between reproductive and vegetative plants, the apparency of vegetative plants was also significantly affected by the diversity of neighbouring community.

After accounting for partial correlations among plant diversity, vegetation characteristics and plant characteristics, the direct negative path linking plant diversity to the abundance of carnivorous arthropods on the vegetative plants remained significant in the SE models. This is in contrast to the 'Associational susceptibility hypothesis' and the 'Enemies hypothesis'. It is possible that this negative direct effect is a result of differences in chemical profiles (e.g. volatile blends) of plots

with different levels of diversity that was not measured here. High levels and complexity of plant odours in more diverse plant communities might hinder parasitoids and predators from detecting the host cues (Wäschke *et al.* 2014). It is important to notice that the method used in our study to collect arthropods might underestimate the number of parasitoids or other visually oriented predators. More studies using other collection methods such as traps are needed to further disentangle the effects of plant diversity on higher trophic level insects associated to individual plants. In agreement with our hypothesis, there was no direct or indirect effect of plant diversity on arthropod abundance on reproductive *J. vulgaris* plants despite the high number of arthropods recorded on these plants. However, there was a strong positive correlation between specialist herbivore abundance and predator abundance. As many of the predators recorded on the flowering plants were ants and the aphid *Aphis jacobaeae* was the most abundant specialised herbivore, this relationship may be explained by the aphid–ant mutualism that is known for this species combination (Vrieling, Smit & Vandermeijden 1991). In this case, the herbivores are not consumed by the predator but are tended for honeydew. The role of such herbivore mutualists have been largely overlooked in biodiversity studies (Moreira *et al.* 2016). In contrast, there were no associations between herbivorous and carnivorous arthropods associated to the vegetative plants, perhaps as a result of the low number of arthropods present on these plants compared to reproductive plants.

Plant quality (primary and secondary compounds) is extremely important in every aspect of insect performance, including host plant selection, growth, survivorship and reproduction (reviewed in Awmack & Leather 2002). In our study, the arthropod abundances associated to focal plants were positively affected by the plant size. This is in accordance with the theory stating that larger plants had higher arthropod abundances (Castagneyrol *et al.* 2013; Schlinkert *et al.* 2015). Interestingly, this pathway was more important for generalist herbivores and predators that are likely more attracted to larger and extra apparent plants and not for specialist herbivores that may use more specific host-related cues. Furthermore, the abundances of generalist herbivores increased and of specialist herbivores decreased with increases in PA concentration of the focal *J. vulgaris* rosettes. This is a surprising result of the SEM analysis and is in contrast to the *specialist–generalist dilemma* (Van der Meijden 1996). We do not have an explanation for this result yet. It contrasts a previous study examining the effects of vegetation complexity on plant chemistry and insect community in grasslands with different land use practices, where the concentration of iridoid glycosides (major defence compounds in *Plantago lanceolata* L.) did not correlate with the abundances of specialist herbivores (Wäschke *et al.* 2015). However, as shown by SEM, the effects of plant diversity on the arthropod abundances in our study were also not mediated by the changes in focal plant chemistry. It is important to mention that the chemistry of the focal plants was measured once during the growing season, whereas the

arthropods were collected four times during the season. Plant chemistry is known to vary with plant seasonal development (Barton & Koricheva 2010) and therefore plant chemistry measured at one time point may not properly reveal the relationship with arthropod abundances of an entire growing season.

In summary, using a field experiment, where plant species diversity was manipulated experimentally, we show that the diversity of the neighbouring vegetation affects the nutritional quality and secondary chemistry of individual plants growing in that community and the abundances of above-ground arthropods that naturally colonise the focal plants. The concentration of the major secondary compounds of the focal plants and the abundance of arthropods decreased with increasing diversity of the neighbouring community. However, the intraspecific variation in plant defence chemistry did not affect arthropod communities associated to the focal plants. Our study emphasises that individual plant–insect interactions should be considered from a community perspective. Future studies should aim at further disentangling the role of plant quality in structuring insect communities in natural settings.

Author's contributions

O.K. and M.B. designed the experiment and collected the data, P.M. performed chemical analyses, O.K. and M.C. performed arthropod identification, O.K. analysed the data and wrote the first draft of the manuscript, and M.B. contributed substantially to revisions.

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Data accessibility

Data deposited in the Marine Data Archive Repository: http://mda.vliz.be/mda/directlink.php?fid=VLIZ_00000241_1394710219 (Kostenko *et al.* 2016a) and in Dryad Digital Repository: http://dx.doi.org/10.5061/dryad.54ht3 (Kostenko *et al.* 2016b).

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Supporting Information

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

Appendix S1. Species mixtures sown in the experimental plots.

Appendix S2. Additional information on pyrrolizidine alkaloids.

Appendix S3. SEM procedure.

Appendix S4. Arthropod community responses.

Appendix S5. Additional data analyses.