RESEARCH ARTICLE

Glyphosate residues in soil can modify plant resistance to herbivores through changes in leaf quality.

S. Ramula[1] (iD), A. Kalske[1], K. Saikkonen[2] & M. Helander[1]

1 Department of Biology, University of Turku, Turku, Finland
2 Biodiversity Unit, University of Turku, Turku, Finland

ABSTRACT

- Glyphosate is the most widely used non-selective herbicide in the world. Glyphosate residues in soil can affect plant quality by modifying plant physiology, hormonal pathways and traits, with potential consequences for plants' interactions with herbivores.
- We explored these indirect effects in the context of plant–herbivore interactions in a perennial, nitrogen-fixing herb. We quantified leaf herbivory for glyphosate-exposed and control plants grown in phosphorus-fertilized and non-fertilized soils, and assessed the impacts of glyphosate treatment on traits related to plant resistance against herbivores (leaf trichome density, leaf mass per area) and performance (aboveground biomass, root:shoot ratio, nodule number, nodule activity). Moreover, we conducted a laboratory feeding experiment to compare the palatability of leaves from glyphosate-exposed and control plants to a generalist mollusc herbivore.
- Herbivore damage and intensity *in situ* increased during the growing season regardless of glyphosate or phosphorus treatment. Glyphosate treatment reduced leaf trichome density but had no effect on the other plant traits considered. Herbivore damage was negatively associated with leaf trichome density. The feeding experiment revealed no difference in the feeding probability of mollusc herbivores between glyphosate-exposed and control plants. However, there was an interaction between glyphosate treatment and initial leaf area for leaf consumption by herbivores: leaf consumption increased with increasing leaf area in both groups, but at a lower rate for glyphosate-exposed plants than for control plants.
- Our results show that glyphosate residues in soil have the potential to indirectly affect aboveground herbivores through changes in leaf quality, which may have mixed consequences for folivore damage.

INTRODUCTION

Glyphosate is the most commonly used herbicide in agriculture and horticulture (Baylis, 2000; Duke & Powles, 2008). Despite claims that it degrades rapidly in ecosystems (Baylis, 2000; Duke & Powles, 2008), glyphosate residues may linger, especially in northern soils, long after application (Laitinen *et al.*, 2009; Helander *et al.*, 2012, 2018). Such residues can affect plant quality by modifying plant hormonal pathways and physiology, with potential consequences for plant–herbivore interactions (Fuchs *et al.*, 2021). More specifically, glyphosate residues may disrupt the biosynthesis of plant defence-related chemical compounds that often play a crucial role in plant attractiveness to herbivores (Fuchs *et al.*, 2021). Such disruptions might result in an increase in plant herbivore load and herbivore damage due to reductions in chemical defence and/or changes in leaf nutritional quality. Indeed, aphids have been found to be more abundant on herbicide-treated, glyphosate-tolerant crop plants than on control crop plants (Dewar *et al.*, 2000; Albajes *et al.*, 2009), although Lipok (2009) reported that aphids preferred non-treated crop plants to glyphosate-treated ones in a food choice experiment. However, previous studies have typically focused on the direct effects of glyphosate-based herbicides on plant–herbivore interactions by observing herbivore abundances and herbivore damage soon after sublethal herbicide application (but see Muola *et al.*, 2021; Fuchs *et al.*, 2022). To date, little is known about the potential indirect effects of glyphosate residues in soil on plant–herbivore interactions (Fuchs *et al.*, 2021).

In addition to potential changes in plant chemical pathways, glyphosate residues can alter plant attractiveness to herbivores through changes in resistance and performance traits. Herbivores rarely consume plants randomly but tend to prefer large and vigorous individuals of high nutritional quality (the plant vigour hypothesis; Price, 1991). In a study of glyphosate-tolerant soybean, direct glyphosate application reduced leaf thickness (Bomfim *et al.*, 2019), making individuals more palatable to folivores. Glyphosate has also been found to reduce nodulation (Reddy & Zablotowicz, 2003; Zobiole *et al.*, 2010), which can be critical for a plant's nitrogen availability and nutrient balance. Moreover, glyphosate residues in soil or in manure fertilizer reduce the growth and reproduction of crop plants (Helander *et al.*, 2019; Soares *et al.*, 2019; Muola *et al.*, 2021) and indirectly reduce plant performance in non-crops through

changes in the soil microbiota (Ramula *et al.*, 2022). On the other hand, the opposite result has also been reported: low doses of glyphosate can induce the growth of both crop and non-crop plants (*e.g.* Helander *et al.*, 2019; Mollaee *et al.*, 2020). Overall, the effects of glyphosate residues on plants likely depend on abiotic conditions, such as the level of soil phosphorus, which interacts with glyphosate in a complex manner (glyphosate competes with phosphorus for binding sites on soil particles; Helander *et al.*, 2012; Kanissery *et al.*, 2019). In general, glyphosate-induced effects on plants, and consequently on herbivores, can be expected to be more pronounced at high levels of soil phosphorus, when the bioavailability of glyphosate is increased, than when phosphorus level is low (Vereecken, 2005; Hebert *et al.*, 2019). The situation is further complicated, however, by the fact that phosphorus itself is able to affect herbivore density through changes in leaf quality. For example, the addition of phosphorus was found to increase insect herbivore density and leaf damage in the invasive grass tall fescue (Graff *et al.*, 2020).

Here, we explore the indirect effects of glyphosate residues in soil on plant–herbivore interactions by using common garden and laboratory experiments with the perennial, nitrogen-fixing herb *Lupinus polyphyllus* (Lindl.). The species is native to western North America and invasive in many European countries, including Finland, where this study was conducted. *Lupinus polyphyllus* contains alkaloids (Wink, 1984; Wink *et al.*, 1995; Kalske *et al.*, 2022), which are considered defence chemicals against herbivores (Wink, 1984) and are further induced by leaf damage (Wink, 1983). We addressed the following three questions: (i) do plants exposed to glyphosate residues in soil experience more leaf herbivory than control plants under common garden or laboratory conditions; (ii) do glyphosate residues modify plant resistance traits (leaf trichome density, leaf mass per area (LMA)) against herbivores and overall performance (aboveground biomass, root:shoot ratio, nodule number, nodule activity); and (iii) how is leaf herbivory *in situ* associated with plant resistance traits? We predicted that plants exposed to glyphosate residues in soil would experience more leaf herbivory than control plants due to reductions in chemical defences, particularly when phosphorus was abundant. On the other hand, reduced nodulation in plants exposed to glyphosate residues could favour lower herbivore attraction if it results in lower nitrogen content in plant tissues. We also predicted that leaf herbivory would be negatively associated with plant resistance traits (leaf trichomes, LMA) and positively associated with plant size, because herbivores tend to prefer large and vigorous individuals (Price, 1991; reviewed in Cornelissen *et al.*, 2008) that are often highly nutritious and/or poorly chemically defended.

MATERIAL AND METHODS

Study species and experimental field

Lupinus polyphyllus (Fabaceae) is a short-lived perennial herb that grows 50–150 cm tall (Fremstad, 2010). As a legume, the species hosts nitrogen-fixing bacteria (rhizobia) in the roots. It inhabits mesic to wet meadows and stream banks in the native range (Beuthin, 2012) and meadows, road verges and various ruderal habitats in the introduced range (Fremstad, 2010). Rosette leaves have long petioles, and each leaf

consists of 10–15 leaflets, each 1–2 cm broad (Fremstad, 2010), which are covered by silky hairs. A flowering plant has one or multiple erect flowering stems, with each containing dozens of small flowers in racemes. The species reproduces mainly from seed (Fremstad, 2010; S. Ramula, personal observation).

In Finland, the species experiences considerably less herbivory than in the native range, where it hosts diverse sucking, mining and chewing herbivores (Kalske *et al.*, 2022). In the study area, we have observed that *L. polyphyllus* is consumed by generalist chewing mollusc herbivores, primarily the land snail, *Arianta arbustorum*, but also by some lepidopteran caterpillars, such as *Vanessa cardui* and *Amphipyra pyramidae*. Pods are occasionally consumed by the gorse shield bug, *Piezodorus lituratus*. We have also detected signs of mammalian herbivores (the European hare, *Lepus europaeus*, and the field vole, *Microtus agrestis*) in the common garden.

In July 2019, we collected seeds from six populations of *L. polyphyllus* along a latitudinal gradient of about 500 km in Finland. In each population, seeds were collected from several haphazardly chosen plants that were at least 2 m apart and were stored in paper bags at room temperature.

The experimental field was established in 2013 at the Ruissalo Botanical Garden (60.4333° N, 22.1733° E) of the University of Turku, Finland, by mixing sand and peat with existing clay soil (pH 7.1) before tilling to a depth of 15 cm. It was fenced with a metal net to keep out large mammalian herbivores. The field contains 22 plots of 23 × 1.5 m each that are 1.5 m apart. Since May 2014, the plots have been tilled with a rotary tiller twice per year (May and October) to a depth of 5 cm and sprayed with 5 l of either tap water (control plots) or tap water mixed with Roundup Gold® (glyphosate concentration 450 g l^{-1}, equivalent to the recommended maximum dose of 3 kg ha^{-1}), using a hand-operated pressure tank (see Helander *et al.*, 2019 for details). Since spring 2018, half of each plot has been fertilized annually with phosphorus (Yara Ferticare™ P-K, 45 kg ha^{-1}) to create phosphorus-fertilized and ambient (non-fertilized) subplots. Crop plants (faba bean, turnip rape, oat, potato) have been cultivated in the plots since 2016.

Herbivory and plant traits in the common garden

In mid-May 2020, we chose 80 fully developed seeds from each plant population for a common-garden experiment. To remove epiphytic microbes, we surface-sterilized the seeds in 0.5% commercial bleach (sodium hypochlorite) solution for 15 min and rinsed them three times with deionized water (Ryan-Salter *et al.*, 2014). We placed hand-scarified seeds on a moist paper towel in foil containers, covered them with plastic film, and left them at room temperature for 4 days. The seedlings that emerged were then planted in plastic trays of 30 × 60 cm that were filled with sterilized potting mix (Kekkilä karkea ruuku-tusseos®, autoclaved at 120°C, 1 bar, 20 min). The seedlings were grown in an unheated greenhouse until early June when they were transplanted into the experimental field (about a week after the end of the safety period of 14 days post-application recommended for Roundup).

We planted six seedlings (one seedling per population, spaced about 15 cm apart) in each phosphorus-fertilized and ambient subplot, resulting in 12 plants in each control or

glyphosate-treated plot, and 264 plants in total. Due to warm weather, 57 seedlings died during the first few days, and we were able to replace about half of them, leaving 241 plants in the experiment. At the same time, we measured plant height to the nearest 0.5 cm and recorded the number of leaves, typically two leaves at that time point (mean = 2.19 ± 0.57 SD). The plants were watered when necessary. To prevent weeds, the space between the experimental plants was covered with 5 cm of hemp bedding and the plots were hand-weeded during the growing season.

We recorded survival, the number of leaves, and height for each individual plant every other week until August (three times in total: late June, mid-July, late July). We quantified leaf damage by chewing (and grazing) herbivores as the proportion of damaged leaves (number of damaged leaves out of all leaves) and scored the intensity of damage as a categorical variable with two levels (< 50% of leaflets damaged = mild damage; ≥ 50% of leaflets damaged = severe damage); herbivore types were not quantified separately. In mid-July, we collected two leaf discs (diameter 7 mm) from each plant to measure herbivore resistance traits. We removed each disc from a separate leaflet of the same leaf (close to the tip of the leaflet to avoid the leaf vein), counted the number of trichomes on the underside of the leaf disc, and estimated leaf mass per unit area (LMA, mg cm^2) as proxy for leaf thickness from dried discs (+65°C for 48 h). To ease comparison among studies, we translated trichome number per leaf disc into number per cm^2. Both leaf trichome density and LMA have been characterized as resistance traits that are inversely correlated with herbivore damage (Hanley *et al.*, 2007; Poorter *et al.*, 2009). At the beginning of August, we harvested the plants, washed the roots, and recorded the number and activity of nodules. We assessed nodule activity visually based on five dissected nodules per plant: red nodules were considered active nitrogen-fixers, while white nodules were considered inactive or dead (Pommeresche & Hansen, 2017). We separated shoot and root biomass and dried them at +65°C for 48 h before weighing and calculating root:shoot ratio (*i.e.* the proportion of root biomass of the total biomass, to describe a plant's resource partitioning). All measurements in the field were conducted by the same individual to reduce variability.

Glyphosate residues in soil

To analyse soil glyphosate concentrations and to verify that control plots were free from glyphosate, we collected soil samples from the experimental field in May 2020, before the glyphosate treatments. The soil samples were sent to the certified laboratory Groen Agro (https://agrocontrol.nl/en/) for the quantification of glyphosate and its main degradation product, aminomethylphosphonic acid (AMPA). Extraction was performed with a mixture of water and acidified methanol, and analyses were conducted using liquid chromatography–tandem mass spectrometry (LC–MS/MS). Separation was performed with a mix mode column using a gradient based on a mixture of water and acetonitrile. The analysis confirmed that the control plots did not contain detectable residues of glyphosate or AMPA at the detection limit of 0.1 mg kg^{-1}, while the glyphosate-treated plots contained 1.51 ± 0.71 mg kg^{-1} glyphosate and 1.35 ± 0.06 mg kg^{-1} AMPA.

Feeding experiment in the laboratory

To compare leaf consumption between glyphosate-exposed and control plants, we conducted a feeding experiment in the laboratory using a generalist grazing herbivore, the land snail (*A. arbustorum*, Helicidae). In mid-July 2020, we collected about 300 snails from a nearby field population of *L. polyphyllus* and kept them in a large, ventilated plastic box in a cool room on standard food (*e.g.* leaves of *Taraxacum* and *Chamaenerion* spp.) for 2 days. The snails had not been exposed to glyphosate-based herbicides in the field population. For the feeding experiment, we chose active snails, measured their shell width, and placed them individually in lidded plastic containers (125 ml, diameter 10 cm) without food for 24 h. A few water drops were added to each container for humidity. The snails were kept at room temperature in an unlit laboratory.

On the morning of the feeding experiment, we collected fully expanded, undamaged leaflets from all available individuals of *L. polyphyllus* in the experimental field (n = 237, one or two leaflets per individual), placed them individually in numbered plastic bags, transported them to the laboratory, and provided them individually to the snails. To measure leaf area at the beginning of the experiment, we photographed the leaflets with a digital camera before providing them to the snails. A leaflet (or two leaflets if small) was placed in the middle of each container with a drop of water and care was taken that the leaflets were not touched by hand throughout the experiment. The snails were allowed to feed for 6.5 h, until late evening before removing them from the containers in chronological order to ensure the same feeding time for all snails. After completing the experiment, we photographed the leaflets again to determine the leaf area consumed (cm^2) by herbivores using the LeafByte app (Getman-Pickering *et al.*, 2020).

Statistical analyses

To explore whether plants exposed to glyphosate residues in soil experienced more leaf herbivory than control plants in the common garden, we conducted a generalized linear mixed model (GLMM) for relative herbivore damage (number of damaged leaves out of all leaves per individual) and herbivore intensity (mild or severe, recorded as a binary variable) using glmmTMB::glmmTMB with a binomial distribution and logit-link function (Brooks *et al.*, 2017) in R software (R 3.5.3; R Development Core Team, 2019). We used glyphosate treatment (glyphosate, control), phosphorus (fertilized, ambient), time (late June, mid-July, late July) and all possible interactions between them as fixed categorical explanatory variables (Table 1). Plant height at a given measurement time was included as a fixed continuous variable to examine the effect of plant size on herbivory. Because height correlated positively with the number of leaves (r = 0.65, *P* < 0.001), we used only the former variable. Subplot nested within plot was included as a random factor, along with plant ID in order to take into account the three sets of repeated measurements taken from each plant. Population was not considered separately due to negligible among-population variation. The intensity of herbivory was assessed based on damaged plants only.

To investigate how glyphosate and phosphorus treatments were associated with plant resistance traits (leaf trichomes,

Table 1. Results of mixed models analysing leaf herbivory (relative damage and intensity of damage) of the perennial herb *Lupinus polyphyllus* exposed to glyphosate residues at different soil phosphorus levels.

Explanatory variable	Damaged leaves (%)		Intensity of damage	
	χ^2	P	χ^2	P
Glyphosate (yes, no)	0.14	0.711	1.16	0.281
Phosphorus (ambient, fertilized)	0.25	0.616	0.686	0.408
Time (3 levels)	**44.35**	**<0.001**	**19.71**	**<0.001**
Plant height	1.10	0.295	**8.12**	**0.004**
Glyphosate × Phosphorus	0.00	0.952	2.21	0.137
Glyphosate × Time	2.75	0.253	1.56	0.459
Phosphorus × Time	2.23	0.327	2.88	0.237
G × P × Time	0.63	0.712	0.68	0.712

Subplot nested within plot and plant ID were used as random factors in both models. Intensity (mild, severe) was assessed from damaged plants only. *P*-values <0.05 are in bold with the degrees of freedom = 1.

LMA) and overall performance (aboveground biomass, root: shoot ratio, number of nodules, nodule viability), we constructed a linear mixed model with restricted maximum likelihood (LMM, lmer4::lmer) for LMA and aboveground biomass (square root-transformed). We used a Poisson GLMM and log-link function for leaf trichomes and the number of nodules, a beta GLMM for root:shoot ratio, and a binomial GLMM for nodule activity (number of red nodules out of all dissected nodules). The two resistance traits were not correlated (r = −0.014, df = 219, $P = 0.841$) and were thus considered separately. We did not analyse survival because only six plants out of 241 died after the first few days of the experiment. In all resistance and performance models, the fixed explanatory variables were glyphosate and phosphorus treatments, and their interaction (Table 2). Plant height at the beginning of the season was included as a covariate, and subplot nested within plot was included as a random factor. Moreover, we used an LMM to explore whether relative herbivore damage *in situ* (proportion of damaged leaves from late July) was associated with the two resistance traits (trichome density, LMA), with subplot nested within plot included as a random factor.

Finally, we conducted a binomial GLMM for feeding probability (a leaflet was consumed = 1 or was intact = 0) and an LMM for leaf area consumed by land snails in the feeding experiment, with glyphosate treatment, phosphorus treatment and their interaction as fixed explanatory variables (Table 3). Subplot nested within plot was used as a random factor. We used snail size (shell width in mm) and initial leaf area as continuous covariates to consider size differences among snails and leaflets. For the LMM, we also included the interaction between initial leaf area and glyphosate treatment because initial leaf area affected differentially leaf consumption for glyphosate-exposed and control plants. To facilitate comparisons within this analysis, we excluded snails that did not consume anything during the experiment (90 snails).

For all LMMs, we verified model assumptions visually from residual plots and transformed the response variable when necessary to improve normality of the residuals (see above for details). For the GLMMs, we explored the residual plots for potential overdispersion and zero inflation using the DHARMa package (Hartig, 2018) and found none. The significance of the fixed variables was evaluated with an *F* test based on the Kenward-Roger method for LMM (lmerTest::anova; Kuznetsova *et al.*, 2017) and with a Wald chi-square test for GLMM (car::Anova; Fox & Weisberg, 2019). All values presented in the figures are back-transformed when necessary.

Table 3. Results of mixed models analysing herbivory of the perennial herb *Lupinus polyphyllus* by the land snail *Arianta arbustorum* in a feeding experiment.

Explanatory variable	Feeding probability		Leaf area consumed	
	χ^2	P	$F_{df,ddf}$	P
Glyphosate (yes, no)	0.83	0.361	$5.43_{1,107}$	**0.022**
Phosphorus (ambient, fertilized)	0.25	0.615	$1.25_{1,18}$	0.278
Initial leaf area	**25.96**	**<0.001**	$41.43_{1,124}$	**<0.001**
Snail size	0.06	0.800	$1.78_{1,132}$	0.184
Glyphosate × Phosphorus	2.05	0.152	$0.66_{1,20}$	0.425
Glyphosate × Leaf area	na		$6.47_{1,125}$	**0.012**

na = not applicable in the final model.
Leaflets were collected from plants exposed to glyphosate residues at different soil phosphorus levels. Subplot nested within plot was used as a random factor. df and ddf denote the degrees of freedom in the numerator and denominator for the LMM, respectively (for the GLMM df is 1). *P*-values <0.05 are in bold.

Table 2. Results of mixed models analysing the effects of glyphosate and phosphorus treatments on herbivore resistance and performance of the perennial herb *Lupinus polyphyllus*.

	Resistance traits				Performance traits							
	Leaf trichomes		LMA		Aboveground biomass (sq rt)		Root:shoot ratio		No. nodules		Nodule activity	
Explanatory variable	χ^2	P	$F_{df,ddf}$	P	$F_{df,ddf}$	P	χ^2	P	χ^2	P	χ^2	P
Glyphosate (yes, no)	**4.93**	**0.027**	$0.60_{1,20}$	0.449	$2.84_{1,20}$	0.108	0.29	0.590	1.40	0.237	0.79	0.373
Phosphorus (ambient, fertilized)	0.05	0.822	$0.00_{1,19}$	0.957	$1.54_{1,20}$	0.229	0.24	0.622	1.03	0.311	0.21	0.645
Initial height	0.10	0.747	$0.04_{1,208}$	0.837	$17.49_{1,213}$	**<0.001**	0.08	0.772	**24.49**	**<0.001**	0.00	0.973
G × P	0.13	0.724	$0.26_{1,19}$	0.616	$0.66_{1,20}$	0.427	0.81	0.367	0.12	0.732	1.66	0.198

Subplot nested within plot was used as a random factor in all models. df and ddf denote degrees of freedom in the numerator and denominator in LMMs, respectively (for GLMMs df is 1). *P*-values <0.05 are in bold.

RESULTS

### Herbivory and plant traits in the common garden

Based on visual assessment, plants in the common garden were consumed by snails and lepidopteran caterpillars but sometimes also by European hares. Herbivory – quantified in terms of the proportion of damaged leaves of *L. polyphyllus* during the growing season – was not associated with glyphosate treatment, soil phosphorus level or plant height (Table 1). The strongest predictor of herbivore damage was time (Table 1); damage increased over the course of the growing season and was more abundant in July than in June (mean = about 30% and 15%, respectively; Fig. 1). Similarly, for plants experiencing herbivory, the intensity of leaf damage increased over time, irrespective of glyphosate and phosphorus treatments, and was more intense in late July than earlier in the growing season (Table 1; mean $\pm$ SE = 0.31 $\pm$ 0.08 in late June, 0.28 $\pm$ 0.06 in mid-July, 0.56 $\pm$ 0.07 in late July). In contrast to our prediction, shorter plants experienced more intense herbivore damage than taller plants (Table 1; intercept = 1.00 and slope $\pm$ SE = -0.089 ± 0.031 for plant height).

With respect to resistance traits, plants in the glyphosate treatment had fewer trichomes than control plants (Table 2, Fig. 2a), while phosphorus treatment had no effect on the resistance traits (Table 2). Leaf herbivory was negatively associated with leaf trichome density (LMM: $F_{1,216} = 4.23$, $P = 0.041$; intercept = 0.265, slope $\pm$ SE = -0.0006 ± 0.0003; Fig. 2b) but was not associated with leaf mass per area (LMM: $F_{1,210} = 2.91$, $P = 0.089$).
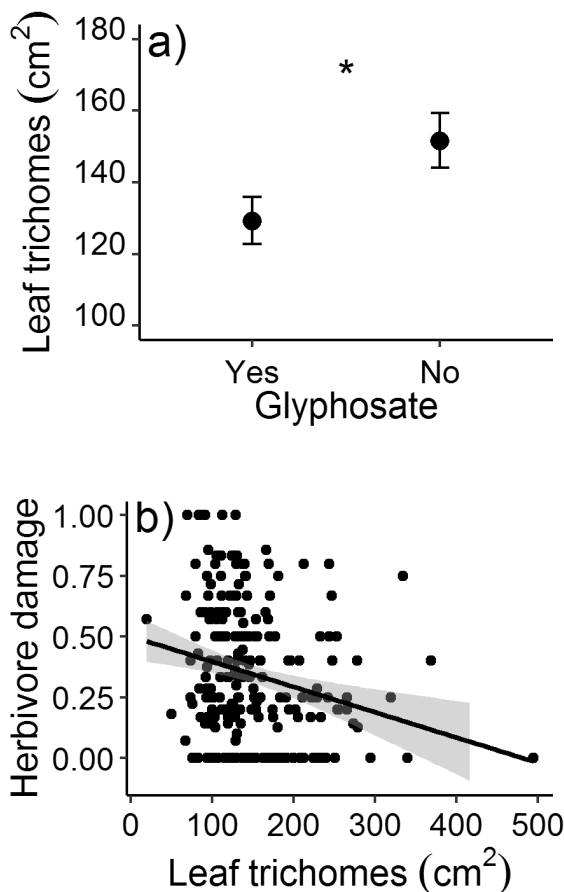
Of the four plant performance traits considered, none was affected by glyphosate or phosphorus treatment on their own or through an interaction (Table 2).

Feeding experiment in the laboratory

Glyphosate or phosphorus treatment had no effect on the feeding probability of land snails in the laboratory, where feeding probability increased with increasing initial leaf area (intercept = 1.398, slope $\pm$ SE = 0.465 $\pm$ 0.092; Table 3). However, the feeding experiment based on the damaged leaflets revealed a significant interaction between glyphosate treatment and
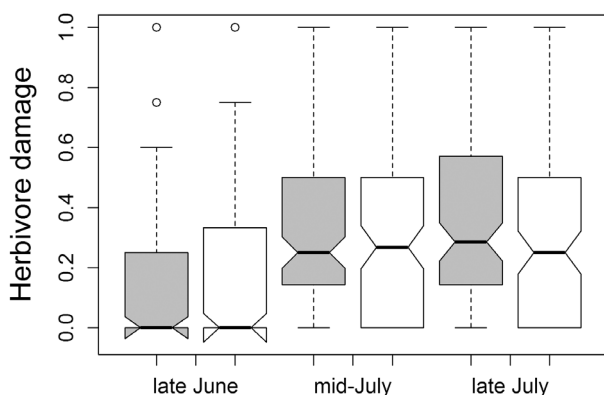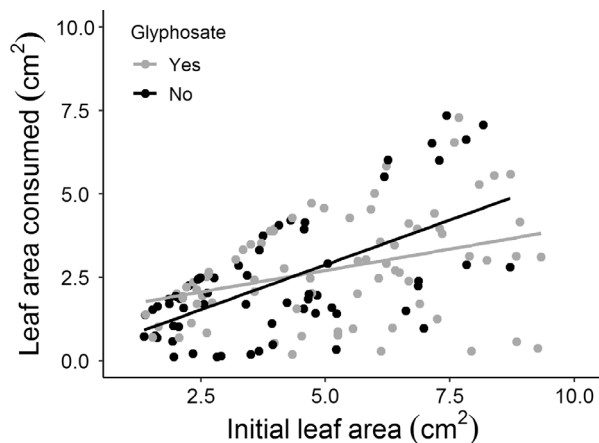

Fig. 2. Number of leaf trichomes for the perennial herb *Lupinus polyphyllus* in relation to (a) glyphosate treatment (mean $\pm$ SE) and (b) herbivore damage *in situ*. Line ($\pm$ SE) was fitted using a linear mixed model, and an asterisk indicates a significant difference between treatments ($P < 0.05$, GLMM).

initial leaf area for the rate of leaf consumption by land snails (Table 3). Leaf consumption increased with increasing initial leaf area, but at a lower rate for glyphosate-exposed plants than for control plants (Fig. 3; slope $\pm$ SE = 0.238 $\pm$ 0.076 and 0.552 $\pm$ 0.119, respectively).

DISCUSSION

Glyphosate residues have been identified as a potential modulator of plant–insect interactions that can alter plant defences against herbivores (Fuchs *et al.*, 2021) or plant nutritional quality (Zobiole *et al.*, 2010; Gomes *et al.*, 2014). We observed that glyphosate residues in soil had no effect on overall leaf herbivory of *L. polyphyllus* in the common garden, despite the fact that glyphosate-exposed plants had lower trichome density. Furthermore, the feeding experiment conducted in the laboratory revealed a negligible effect of glyphosate residues on the feeding probability of the generalist mollusc herbivore. However, leaf consumption differed between glyphosate-exposed and control plants relative to initial leaf area based on the data containing damaged leaflets. The mollusc herbivore consumed leaflets of glyphosate-exposed plants at a lower rate relative to their area than those of control plants. This observation is probably an artefact caused by the limited amount of food

Fig. 1. Herbivore damage quantified as the proportion of damaged leaves of glyphosate-exposed (grey) and control (white) individuals of the perennial herb *Lupinus polyphyllus* during the growing season. Notches show 95% confidence intervals around the median.

Fig. 3. Leaf consumption by the land snail *Arianta arbustorum* in relation to initial leaf area for glyphosate-exposed and control individuals of the perennial herb *Lupinus polyphyllus*. The lines were fitted from a general linear model and error bars are omitted for clarity.

provided to the herbivores in the experiment, although it might also reflect differential preferences for the two types of leaflets.

In contrast to our prediction, neither herbivore damage nor the intensity of damage differed between glyphosate-exposed and control plants during the growing season. This result contradicts that found by Helander *et al.* (2019), who reported that oats exposed to glyphosate residues in soil experienced more grazing by barnacle geese than untreated control oats. The discrepancy between the two studies might be due to the different plant species examined (monocots *versus* eudicots) and/or the use of different herbivores with different feeding types (grazing vertebrates *versus* chewing invertebrates) and food plant preferences. Monocots and eudicots do not systematically differ in sensitivity to sublethal doses of glyphosate-based herbicides (reviewed in Cederlund, 2017), but plant hormonal responses to glyphosate residues in soil seem to be species specific (Fuchs *et al.*, 2022), with diverse consequences for herbivores. Grazing vertebrate herbivores, particularly geese, may choose food through tasting and visual cues (*e.g.* Wink *et al.*, 1993; Hassall *et al.*, 2001), preferring shorter plants over taller ones (Hassall *et al.*, 2001), while invertebrate herbivores often use olfactory cues to locate a host plant (Szendrei & Rodriguez-Saona, 2010; Kiss, 2017). As in the present study, Muola *et al.* (2021) observed no difference in the amount of chewing insect herbivore damage between glyphosate-exposed and control plants in two strawberry (*Fragaria*) species. Likewise, a sublethal dose of glyphosate-based herbicide did not dramatically affect leaf damage by biocontrol weevils in water hyacinth, *Eichhornia crassipes* (Katembo *et al.*, 2013). Overall, the strongest predictor of both herbivore damage and herbivore intensity in the present study was time; leaf herbivory increased during the growing season in all groups. This finding is not surprising, because in perennial herbs with longer leaf lifespans, herbivore damage accumulates until leaves are wilted and shed or the plant dies back.

Interestingly, although glyphosate treatment did not affect leaf herbivory *in situ*, we did observe that plants exposed to glyphosate residues had lower trichome density. As trichome

development is regulated by phytohormones, particularly jasmonic and gibberellic acids (Khan *et al.*, 2021), lower trichome density might be due to physiological and hormonal changes in plants induced by glyphosate residues (Gomes *et al.*, 2014; Fuchs *et al.*, 2021). Non-glandular leaf trichomes generally function as a mechanical defence against folivores, particularly against molluscs and chewing and sucking herbivores (reviewed in Hanley *et al.*, 2007), while glandular trichomes are fundamental in chemical defence. Indeed, we found here that relative herbivore damage was negatively associated with trichome density: herbivores were more likely to attack plants with fewer leaf trichomes. Herbivore damage in the common garden was not associated with leaf mass per area (LMA), suggesting that LMA did not act as a mechanical defence for *L. polyphyllus* in this case. This conflicts with our previous finding, in the same study species, that leaf area eaten by snails in a feeding experiment correlated negatively with LMA at the population level (Kalske *et al.*, 2022). However, LMA has multiple functions, many of which are associated with photosynthetic capacity and plant vigour (Poorter *et al.*, 2009). These other functions might have been more important in the present common garden study than a function as a mechanical defence.

Neither glyphosate nor phosphorus treatment had effects on the performance of the study species. This result is in line with our previous findings for the same study system (Ramula *et al.*, 2022), confirming that *L. polyphyllus* is not particularly sensitive to glyphosate residues or phosphorus levels in soil. Such robustness to glyphosate residues may provide a competitive advantage over other, more sensitive, herbaceous species in the agricultural or urban environments where herbicides are used. Our results are also consistent with a previous study that reported a high tolerance of the native perennial *Lupinus albicaulis* to sublethal doses of glyphosate-based herbicide (Olszyk *et al.*, 2013). Although phosphorus levels were not explicitly determined during the study, we assumed that phosphorus fertilization using the manufacturer's recommended dose would have increased soil phosphorus concentration compared to non-fertilized soil.

To conclude, based on repeated applications of glyphosate-based herbicide over multiple years, our results show that glyphosate residues in soil decrease leaf trichome density. If this decrease is a prevalent effect, glyphosate residues in soil might be expected to reduce plant resistance to herbivores. Such reduced resistance against invertebrate folivores could enhance the control of unwanted non-crop species by increasing their vulnerability to herbivores, but might be harmful for cultivated crop species. Surprisingly, folivores do not seem to be particularly attracted to glyphosate-exposed plants despite their lower leaf trichome density.

ACKNOWLEDGEMENTS

CONFILCTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

All authors designed the study, SR and AK conducted the experiments, SR performed statistical analyses and produced the first draft of the manuscript, all authors reviewed and edited the text.

DATA AVAILABILITY STATEMENT

The datasets generated and analysed during the study are available from the Dryad Digital Repository, https://doi.org/10.5061/dryad.qfttdz0kn.

REFERENCES

Albajes R., Lumbierres B., Pons X. (2009) Responsiveness of arthropod herbivores and their natural enemies to modified weed management in corn. *Environmental Entomology*, **38**, 944–954.

Baylis A.D. (2000) Why glyphosate is a global herbicide: Strengths, weaknesses and prospects. *Pest Management Science*, **56**, 299–308.

Beuthin, M. (2012) Plant guide for bigleaf lupine (Lupinus polyphyllus). USDA-Natural Resources Conservation Service, Plant Materials Center, Corvallis, OR, USA.

Bomfim N.C.P., Silva M.S., Camargos L.S., Martins A.R. (2019) Ultrastructural and histochemical changes in glyphosate-tolerant soybean leaves exposed to glyphosate. *Journal of Agricultural Science*, **11**, 243–256.

Brooks M.E., Kristensen K., van Benthem K.J., Magnusson A., Berg C.W., Nielsen A., Skaug H.J., Maechler M., Bolker B.M. (2017) glmmTMB balances speed and flexibility among packages for zero-inflated generalized linear mixed modeling. *The R Journal*, **9**, 378–400.

Cederlund H. (2017) Effects of spray drift of glyphosate on nontarget terrestrial plants – A critical review. *Environmental Toxicology and Chemistry*, **36**, 2879–2886.

Cornelissen T., Fernandez G.W., Vasconcellos-Neto J. (2008) Size does matter: variation in herbivory between and within plants and the plant vigor hypothesis. *Oikos*, **117**, 1121–1130.

Dewar A.M., Haulock A.L., Bean K.M., May M.J. (2000) Delayed control of weeds in glyphosate-tolerant sugar beet and the consequences on aphid infestation and yield. *Pest Management Science*, **56**, 345–350.

Duke S.O., Powles S.B. (2008) Glyphosate: A once-in-a-century herbicide. *Pest Management Science*, **64**, 319–325.

Fox J., Weisberg S. (2019) *An R companion to applied regression*, 3rd edition. Sage, Thousand Oaks CA, USA.

Fremstad, E. (2010) NOBANIS – Invasive alien species fact sheet – Lupinus polyphyllus. In: Online Database of the European Network on Invasive Alien Species NOBANIS. Available at: www.nobanis.org. Accessed 11 November 2021.

Fuchs B., Laihonen M., Muola A., Saikkonen K., Dobrev P.I., Vankova R., Helander M. (2022) A glyphosate-based herbicide in soil differentially affects hormonal homeostasis and performance of non-target crop plants. *Frontiers in Plant Science*, **12**, 787958.

Fuchs B., Saikkonen K., Helander M. (2021) Glyphosate-modulated biosynthesis driving plant defense and species interactions. *Trends in Plant Science*, **26**, 312–323.

Getman-Pickering Z.L., Campbell A., Aflitto N., Grele A., Davis J.K., Ugine T.A. (2020) LeafByte: a mobile application that measures leaf area and herbivory quickly and accurately. *Methods in Ecology and Evolution*, **11**, 215–221.

Gomes M.P., Smedbol E., Chalifour A., Henault-Ethier L., Labrecque M., Lepage L., Lucotte M., Juneau P. (2014) Alteration of plant physiology by glyphosate and its by-product aminomethylphosphonic acid: An overview. *Journal of Experimental Botany*, **65**, 4691–4703.

Graff P., Gundel P.E., Salvat A., Cristos D., Chaneton E.J. (2020) Protection offered by leaf fungal endophytes to an invasive species against native herbivores depends on soil nutrients. *Journal of Ecology*, **108**, 1592–1604.

Hanley M.E., Lamont B.B., Fairbanks M.M., Rafferty C.M. (2007) Plant structural traits and their role in anti-herbivore defence. *Perspectives in Plant Ecology, Evolution and Systematics*, **8**, 157–178.

Hartig, F. (2018) DHARMa: Residual diagnostics for hierarchical (multi-level / mixed) regression models. R package version 0.2.0

Hassall M., Riddington R., Helden A. (2001) Foraging behaviour of brent geese, *Branta b. bernicla*, on grasslands: effects of sward length and nitrogen content. *Oecologia*, **127**, 97–104.

Hebert M.P., Fugere V., Gonzalez A. (2019) The overlooked impact of rising glyphosate use on phosphorus loading in agricultural watersheds. *Frontiers in Ecology and the Environment*, **17**, 48–56.

Helander M., Pauna A., Saikkonen K., Saloniemi I. (2019) Glyphosate residues in soil affect crop plant germination and growth. *Scientific Reports*, **9**, 19653.

Helander M., Saloniemi I., Omacini M., Druille M., Salminen J.P., Saikkonen K. (2018) Glyphosate decreases mycorrhizal colonization and affects plant–soil feedback. *Science of the Total Environment*, **642**, 285–291.

Helander M., Saloniemi I., Saikkonen K. (2012) Glyphosate in northern ecosystems. *Trends in Plant Science*, **17**, 569–574.

Kalske, A., Luntamo, N., Salminen, J-P., Ramula, S. (2022) Introduced populations of the garden lupine are adapted to local generalist snails but have lost alkaloid diversity. *Biological Invasions*, **24**, 51–65.

Kanissery R., Gairhe B., Kadyampakeni D., Batuman O., Alferez F. (2019) Glyphosate: its environmental persistence and impact on crop health and nutrition. *Plants*, **8**, 499.

Katembo N., Hill M.P., Byrne M.J. (2013) Impacts of a sub-lethal dose of glyphosate on water hyacinth nutrients and its indirect effects on Neochetina weevils. *Biocontrol Science and Technology*, **23**, 1412–1426.

Khan R.A., Mohammad H.I.M., Muzafar S., Jan S., Abbas N. (2021) Transcriptional regulation of trichome development in plants: an overview. *Proceedings of the Indian National Science Academy*, **87**, 36–47.

Kiss T. (2017) Do terrestrial gastropods use olfactory cues to locate and select food actively? *Invertebrate Neuroscience*, **17**, 9.

Kuznetsova A., Brockhoff P.B., Christensen R.H.B. (2017) lmerTest package: tests in linear mixed effects models. *Journal of Statistical Software*, **82**, 1–26.

Laitinen P., Rämö S., Siimes K. (2009) Glyphosate and phosphorus leaching and residues in boreal sandy soil. *Plant and Soil*, **323**, 267–283.

Lipok J. (2009) Dual action of phosphonate herbicides in plants affected by herbivore –model study on black bean aphid Aphis fabae rearing on broad bean Vicia faba plants. *Ecotoxicology and Environmental Safety*, **72**, 1701–1706.

Mollaee M., Mobli A., Thompson M., Chauhan B.S. (2020) Responses of glyphosate-resistant and susceptible biotypes of Echinochloa colona to low doses of glyphosate in different soil moisture conditions. *PLoS One*, **15**, e0233428.

Muola A., Fuchs B., Laihonen M., Rainio K., Heikkonen L., Ruuskanen S., Saikkonen K., Helander M. (2021) Risk in the circular food economy: Glyphosate-based herbicide residues in manure fertilizers decrease crop yield. *Science of the Total Environment*, **750**, 141422.

Olszyk D., Blakeley-Smith M., Pfleeger T., Lee E.H., Plocher M. (2013) Effects of low levels of herbicides on prairie species of the Willamette Valley, Oregon. *Environmental Toxicology and Chemistry*, **32**, 2542–2551.

Pommeresche, R., Hansen, S. (2017) Examining root nodule activity on legumes. Technical Note. Research Institut of Organic Agriculture (FiBL), CH-Frick and Norwegian Centre for Organic Agriculture (NORSØK), NO-Tingvoll.

Poorter H., Niinemets Ü., Poorter L., Wright I.J., Villar R. (2009) Causes and consequences of variation in leaf mass per area (LMA): A meta-analysis. *New Phytologist*, **182**, 565–588.

Price P.W. (1991) The plant vigor hypothesis and herbivore attack. *Oikos*, **62**, 244–251.

R Development Core Team (2019) *R: A language and environment for statistical computing*. R Foundation for Statistical Computing, Vienna, Austria. https://www.R-project.org/

Ramula S., Mathew S.A., Kalske A., Nissinen R., Saikkonen K., Helander M. (2022) Glyphosate residues alter the microbiota of a perennial weed with a minimal indirect impact on plant performance. *Plant and Soil*, **472**, 161–174.

Reddy K.N., Zablotowicz R.M. (2003) Glyphosate-resistant soybean response to various salts of glyphosate and glyphosate accumulation in soybean nodules. *Weed Science*, **51**, 496–502.

Ryan-Salter T.P., Black A.D., Andrews M., Moot D.J. (2014) Identification and effectiveness of rhizobial strains that nodulate *Lupinus polyphyllus*. *Proceedings of the New Zealand Grassland Association*, **76**, 61–66.

Soares C., Pereira R., Spormann S., Fidalgo F. (2019) Is soil contamination by a glyphosate commercial formulation truly harmless to non-target plants? – Evaluation of oxidative damage and antioxidant responses in tomato. *Environmental Pollution*, **247**, 256–265.

Szendrei Z., Rodriguez-Saona C. (2010) A meta-analysis of insect pest behavioral manipulation with plant volatiles. *Entomologia Experimentalis et Applicata*, **134**, 201–210.

Vereecken H. (2005) Mobility and leaching of glyphosate: A review. *Pest Management Science*, **61**, 1139–1151.

Wink M. (1983) Wounding-induced increase of quinolizidine alkaloid accumulation in lupin leaves. *Zeitschrift für Naturforschung C*, **38**, 905–909.

Wink M. (1984) Chemical defense of lupins. Mollusc-repellent properties of quinolizidine alkaloids. *Zeitschrift für Naturforschung C*, **39**, 553–558.

Wink M., Hofer A., Bilfinger M., Englert E., Martin M., Schneider D. (1993) Geese and dietary allelochemicals – Food palatability and geophagy. *Chemoecology*, **4**, 93–107.

Wink M., Meißner C., Witte L. (1995) Patterns of quinolizidine alkaloids in 56 species of the genus *Lupinus*. *Phytochemistry*, **38**, 139–153.

Zobiole, L.H.S., de Oliveira, RS Jr., Huber, D.M., Constantin, J., de Castro, C., de Oliveira, F.A., de Oliveira, A. Jr (2010) Glyphosate reduces shoot concentrations of mineral-nutrients in glyphosate-resistant soybeans. *Plant and Soil*, **328**, 57–69.