Escaping herbivory through chemical novelty? Field performance and herbivore attack in the cardenolide-producing crucifer plant *Erysimum cheiranthoides*

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Abstract

1. Plants in the Brassicaceae produce glucosinolates as potent defences against generalist herbivores, but many specialists have evolved the ability to tolerate or even deactivate these compounds. As a possible co-evolutionary response to attack by specialists, plants in the genus *Erysimum* gained the ability to produce cardenolide defences in addition to glucosinolates.
2. We grew the dually defended *E. cheiranthoides* and the glucosinolate-defended *Brassica nigra* side-by-side in agricultural fields surrounded by other Brassicaceae crops. Over the course of two field seasons, we monitored focal plants to characterise their associated

herbivore community, and we quantified herbivore damage and measured plant growth. In the second year, we additionally manipulated herbivore density and plant competition to identify direct and indirect effects on plant fitness.

3. Both plant species were attacked by a diverse community of specialist herbivores already present on surrounding Brassicaceae crops. Most herbivores strongly favoured *B. nigra*, reaching higher densities and causing intense damage that largely suppressed growth of this plant. The same herbivores attacked *E. cheiranthoides*, but at much lower densities that caused little damage and did not impact plant growth.
4. In accordance with differential susceptibilities to herbivores, herbivore removal benefitted *B. nigra* by reducing damage and increasing plant growth, while it had no positive effect on *E. cheiranthoides*. In fact, herbivore removal had an indirect negative effect on *E. cheiranthoides* when growing in conspecific pairs, as these plants suffered from increased competition by previously herbivore-suppressed *B. nigra*.

Synthesis: Our results agree with a successful escape of *E. cheiranthoides* from specialist herbivory, as even though plants are still attacked when herbivores are abundant, these herbivores cause little damage and cause no reduction in fitness. However, the escape from herbivory has likely come at loss of competitive ability in the absence of herbivores, which would explain why despite its key innovation, *E. cheiranthoides* has been unable to gain dominance in any plant community.

1. Introduction

In nature, plants are constantly threatened by herbivores, which have the potential to alter plant evolution and cause substantial yield loss in agriculture (Johnson, 2011). To preserve their limited resources, plants have gradually evolved several layers of defence, including constitutively expressed physical barriers, chemical defences, and inducible molecular responses (War et al., 2012). Chemical compounds play a central role in plant-herbivore interactions, as they can shape the community of herbivores that attack a plant, and in turn are shaped by herbivore-imposed selection (Agrawal,

Hastings, et al., 2012; Züst et al., 2012). As a consequence of such reciprocal adaptations, plants have evolved a vast diversity of toxic, repellent, or antinutritive compounds (Speed et al., 2015).

Phytochemical diversity is thought to be at least in parts the result of an ongoing arms race between plants and their natural enemies (Ehrlich & Raven, 1964). The gain of novel defences enables plants to temporarily escape their herbivores and evolve in enemy-free space, which can result in increased rates of plant speciation ("escape and radiate", Ehrlich & Raven, 1964; Maron et al., 2019). However, over time some herbivores inevitably overcome these defences through specialised adaptations, which may in turn result in increased rates of speciation for resistant herbivore lineages. For example, plants in the Brassicales evolved potent glucosinolate (GSL) defences approximately 80 million years ago (Edger et al., 2015), but since then, several specialist herbivores evolved strategies to disarm this defence, and even make use of the defensive compounds as host-finding cues or for sequestration (Aliabadi et al., 2002; Ratzka et al., 2002; Sporer et al., 2021; Steward & Boggs, 2020). In fact, a phylogenetic comparison between Brassicales and associated herbivorous Pierinae butterflies revealed that gains of structural novelties in glucosinolate defences initially accelerated speciation rates in plants, but each was followed by shifts in herbivore speciation rates as they subsequently adapted to these new plant defences (Edger et al., 2015). In consequence, extant species of the Brassicaceae commonly produce a diverse arsenal of GSL defences, but they are nonetheless attacked by a diverse community of GSL-resistant specialised herbivores.

Phytochemical novelty mostly arises from neofunctionalization of existing biosynthetic enzymes (Moghe & Last, 2015), rather than from the gains of entire new pathways. Phytochemical diversity is therefore often limited by biosynthetic and phylogenetic constraints and as a result, related plant species often share functionally similar defensive chemicals (Rønsted et al., 2012). Such limited phytochemical diversity may facilitate and/or accelerate the evolution of tolerance among specialised herbivores, and likely results in diminishing returns of incremental phytochemical diversification. In order to escape their coevolved herbivores, plants thus must occasionally overcome these constraints and evolve new biosynthetic pathways and functionally novel compounds as a "second line of defence" against specialised enemies (Feeny, 1977). Several members of the Brassicaceae apparently have gained such novel defences in addition to their ancestral GSL defences, including *Barbarea*

species which evolved the ability to produce evolutionary novel saponins (Shinoda et al., 2002), *Cochlearia officinalis* which produces novel alkaloids (Brock et al., 2006), and plants in the genus *Erysimum* which are able to synthesise novel cardenolides (Makarevich et al., 1994).

The genus *Erysimum* is ideally suited to study coevolutionary escape from specialist herbivores, as cardenolides are a potent class of toxins with distinctly different mode of action from GSLs (Agrawal, Petschenka, et al., 2012), and at least some GSL-resistant herbivores are completely repelled from *Erysimum* plants by these compounds (Sachdev-Gupta et al., 1993). Cardenolides evolved in the genus *Erysimum* only within the last 5 million years, and their gain was followed by rapid diversification and speciation (Züst et al., 2020), matching a renewed pattern of 'escape and radiate'. Most *Erysimum* species co-express GSL and cardenolide defences (Züst et al., 2020), with the GSL defences likely keeping generalist herbivores at bay, while the novel cardenolide defences may have specifically evolved to deter GSL-resistant Brassicacaeae herbivores. To the best of our knowledge, no herbivore has yet evolved a specialised resistance to cardenolides in the *Erysimum* system, and only one cardenolide-specialised seed bug has shifted its host range to include seeds from these plants (Petschenka et al., 2022). The *Erysimum* system thus offers a unique opportunity for studying a coevolutionary system where the herbivores are currently trailing behind in adaptation.

Here we use the annual plant *Erysimum cheiranthoides* to characterise the natural community of herbivores that attacks this plant in central Europe. We aim to address whether the gain of cardenolides has truly allowed this plant to escape from herbivory in a natural community, and to understand the selective forces that remain acting on this species in its putative 'enemy-free space'. To this end we grew the dually-defended *E. cheiranthoides* side-by-side with glucosinolate-defended *Brassica nigra* over two consecutive years. Plants were grown in fields adjacent to fields of oilseed rape (*Brassica napus*), which provided a high density of herbivores specialized on Brassicaceae. We monitored herbivore abundance and plant damage for a full growing season in each year, and in the second year we additionally manipulated herbivore density and plant competition. We hypothesise that due to its novel cardenolide defence, *E. cheiranthoides 1)* is better defended and damaged less by herbivores, and *2)* consequently achieves a higher fitness compared with *B. nigra*, but *3)* may be competitively inferior to *B. nigra* when herbivores are absent.

2. Methods

2.1 Plant material

Plants used in this study were grown from seed and subsequently transplanted to field sites for monitoring plant performance and herbivore attack. We selected field sites adjacent to crops of oilseed rape (*Brassica napus*) to ensure high pressure from specialized herbivores. We compared the focal *Erysimum cheiranthoides* to *Brassica nigra* as a control because both are annual plants with similar life cycles, and *B. nigra* is well-liked by many GSL-resistant herbivores. For *E. cheiranthoides*, plants were grown from seeds of 39 natural accessions in the first year, and a subset of 9 accessions in the second year. Accessions were originally collected from different locations across Europe and propagated in a common greenhouse environment for two generations. For the current study we focus on species-level patterns and treat accessions of *E. cheiranthoides* as equivalent. Seeds of *B. nigra* used in this study were originally provided by the Botanical Garden of Krefeld, Germany.

2.2 Field experiments

2.2.1 Experiment 1: 2022 herbivory

Plants were sown in late April for transplantation in late May. We soaked seeds of *E. cheiranthoides* in water and kept them at 4 °C for three days to break dormancy. Three soaked seeds were placed on the soil surface of pots (7 × 7 × 8 cm) filled with a 1:1 mixture of washed sand and peat-based planting substrate (Einheitserde, Patzer Erden GmbH, Germany). Seeds of *B. nigra* were directly sown into pots without pre-treatment and lightly covered with soil. All pots were moved to a growth chamber (constant light, 23 °C, 60% relative humidity) and covered with transparent plastic hoods for one week to maintain high humidity during seed germination. Seven days after sowing, plants were moved to an open greenhouse and shaded from direct sunlight for continued growth and hardening. In the last week of May, three-week old plants were transplanted into a freshly tilled field at the Swiss federal agricultural research station (Agroscope Reckenholz, Switzerland). Plants were transplanted to

the field inside their plastic pot to slow the escape of growing roots and To limit excessive biomass growth in the field. Plants were watered in the field as needed throughout the experiment.

In 2022, we used a rectangular field site measuring 6 m × 80 m in a SW to NE orientation (Figure S1a, 47°26'15.5"N 8°31'36.5" E), located alongside a larger field of oilseed rape (*Brassica napus*). On either side of the experimental plot, 1 m-wide buffer strips of a commercial cover crop mixture consisting of *Raphanus sativus* and *Avena 6trigose* had been sown two months earlier. We overlaid a 6 × 80 grid to the experimental plot, with grid lines spaced 1 m apart and with a 0.5 m distance to the plot edge on each side. Each node in the grid was assigned a unique position and consecutive sets of eight rows were assigned to ten blocks (see Figure S1a). Within each block, four *B. nigra* plants were randomly assigned to a grid position. The remaining positions were filled with 39 *E. cheiranthoides* plants, leaving 4-6 positions empty per block. At the field scale, this resulted in 379 *E. cheiranthoides* plants and 40 *B. nigra* plants in total. Throughout the experiment, the experimental plot was weeded regularly and kept free of other vegetation. Unfortunately, cover strips had to be mowed halfway through the experiment (week 5) to prevent weed seed dispersal.

We closely monitored plants over a period of nine weeks. In weeks 3, 6, and 9, we assessed plant damage by herbivores, and in the six remaining weeks, we recorded the herbivores that were present on each focal plant. In weeks of herbivore community assessments, we visited each plant once and recorded the type and number of herbivores sitting or feeding on the plant. Herbivores were recorded at species level where visual identification was feasible, and as functional groups otherwise (e.g., flea beetles, weevils). Assessment of all plants was completed over 2-3 subsequent days in each week. In weeks of damage assessment, we visited each plant once to record leaf damage and plant size. We approximated total damage by counting the number of leaves on a plant with visible herbivore damage of any size, and we approximated plant size by counting the total number of leaves, as well as measuring the height of the plant stem from root base to apical meristem.

A representative sample of leaves were sequentially harvested and analysed to determine nutritional quality of plants throughout the 2022 experiment (Supplementary Methods). Finally, whole plants were sequentially harvested at different times of the experiment. Plants in blocks 1-3 were harvested in week 8 to capture early plant biomass, mostly before seed setting. Plants in blocks 4-6

were harvested in week 11, and plants in blocks 8-10 were harvested in week 14. A few plants had stopped vegetative growth and completely ripened their seed earlier, thus, to prevent loss of biomass we harvested these individuals ahead of schedule. For biomass harvesting, we cut the stem of each plant at soil level and placed the entire aboveground biomass in paper bags. Aboveground biomass was dried at 60°C for 48-96 hours and weighed to the nearest g.

Plants in block 7 were dug out of the soil in week 8 and transported back to a greenhouse where we let them finish their development and ripen their seeds. At the onset of seed ripening, each plant was wrapped in a paper bag to prevent seed loss. After harvesting, seeds were separated from vegetative biomass, biomass was dried and weighed, and seed mass was weighed separately for each plant in this block. Finally, we counted and weighed a subset of 100 seeds for each plant to determine average seed size.

2.2.2 Experiment 2: 2023 herbivory and herbivore exclusion

Based on the results of the 2022 field experiment, we set up a modified field experiment in 2023. We used fewer individual plants but added treatments of plant competition and herbivore suppression. To test for the effect of plant-plant competition, a subset of *E. cheiranthoides* plants was grown together with a *B. nigra* plant in the same 7 x 7 x 8 cm pot. To reduce herbivore loads, half of all plants (individual and competing) were treated with weekly applications of lambda-cyhalothrin (Kendo® Gold, Maag Gardens, Switzerland), a non-systemic pyrethroid insecticide. Plants were grown from seed in a growth chamber and moved to a greenhouse as in 2022, and after three weeks of growth transplanted to a field site at Agroscope Reckenholz in the last week of May.

In 2023, we used a different rectangular field site measuring 4 m × 66 m in a W to E orientation (Figure S1b, 47°25’42.1”N 8°31’23.5”E), again located alongside a larger field of oilseed rape (*Brassica napus*). On either side of the experimental plot, 1.5 m-wide buffer strips of the same cover crop mixture had been sown one week prior to transplanting, which were maintained throughout the experiment. We overlaid six 4 x 10 grids to the experimental plot, with grid lines spaced 1 m apart and with a 0.5 m distance to the plot edge on each side. Each grid corresponded to a block, separated from the next block by 1.5 m. Individual plants were grown in blocks 1, 2, 5, and 6, while pairs of

competing plants were grown in blocks 3 and 4 (Figure S1b). Within each block of individual plants, four replicates of *B. nigra* and 36 *E. cheiranthoides* plants (9 accessions x 4 replicates) were assigned to the grid nodes following a stratified random design to avoid spatial clustering of *B. nigra* plants. Within the two blocks of competing plants, four to five replicates for each of the 9 *E. cheiranthoides* accessions, each paired with one *B. nigra* plant, were randomly assigned to the grid nodes. At the field scale, this resulted in 16 *B. nigra* and 144 *E. cheiranthoides* plants growing individually, and 80 pairs of competing *B. nigra* and *E. cheiranthoides*. Starting at transplanting, we applied weekly sprays of lambda-cyhalothrin (15 mg/L water) to all plants in blocks 2, 4, and 6, completely wetting all leaf surfaces. We maintained spraying for seven consecutive weeks, increasing the total volume sprayed as plants were growing larger, from approximately 30 mL per plant in the first three weeks up to 150 mL per plant by week 7. Plants in blocks 1, 3, and 5 were sprayed with an equivalent amount of water.

We closely monitored individual plants over a period of nine weeks. We assessed plant damage in weeks 3, 6, and 9, and recorded herbivore presence in the six remaining weeks. Herbivore community assessments were carried out as before, but we modified our damage assessment. In weeks of plant damage assessments, we visited each plant once and counted the number of damaged leaves, separately counting leaves with high (leaves with more than 75% of area damaged) and low damage. At the same time, we measured size as plant height and the total number of leaves. However, total leaf counts had high levels of observer bias for older plants in 2022, thus for older plants (weeks 6 and 9) we instead measured plant diameter and branch number. We additionally counted the total number of leaves on 20 randomly selected plants in weeks 6 and 9, and from this subset generated a predictive linear model of total leaf number as a function of plant height, diameter, branch number, assessment week and observer. We then used this model to predict total number of leaves for all remaining plants. In the 2023 field experiment, all plants were harvested in week 10. We cut plants at soil level and placed the entire aboveground biomass in paper bags, followed by drying at 60°C for 48-96 hours and weighing to the nearest gram.

2.3 Statistical analyses

The current study is focused on differences between *E. cheiranthoides* and *B. nigra*, while variation among *E. cheiranthoides* accessions will be presented elsewhere. For the 2022 field experiment, we use herbivore community, damage, and plant size data from all 40 *B. nigra* plants and 40 neighbouring *E. cheiranthoides* plants. Neighbours were selected randomly out of the maximum of four immediate *B. nigra* neighbours without repeat sampling (Figure S1a). For the smaller 2023 field experiment, we use data from all 16 individual *B. nigra* plants and 32 neighbouring individual *E. cheiranthoides* plants. Two neighbours were selected randomly out of the maximum of four immediate *B. nigra* neighbours without repeat sampling to avoid issues with small sample sizes (Figure S1b). We also present biomass data from all 80 plant pairs in the competition treatment. All analyses were performed in the statistical software R v.4.3.1 (R Core Team, 2023).

2.3.1 Herbivore community composition and phenology

We first summarized herbivore communities as the total number of individuals observed per plant across the season. We compared the mean herbivore abundances across entire seasons between plant species (for 2022), and between plant species and insecticide treatments (for 2023) using separate negative-binomial Generalized Linear Models (GLM, function 'glm.nb' in the *MASS* package, (Venables & Ripley, 2002)).

To examine temporal variation in herbivore presence and abundance, we employed a series of Generalised Additive Mixed-Effects Models (GAMMs) using the 'gamm' function in the *mgcv* package (Wood et al., 2016). We first fit separate negative binomial GAMMs to total herbivore counts in 2022 and 2023. For the 2022 data, we constructed a full model that included a parametric term of 'plant species' and a smoothing term (based on thin plate regression splines) for 'days in the field' that was allowed to vary by plant species (i.e. separate smoothers for each plant species). We determined the number of nodes (parameter *k* in GAMM) by subtracting 1 from the total number of weeks insects were observed in the field. Plant identity was included as a random effect to account for repeated observations of the same individual. To estimate the significance of the parametric term we used the default Wald tests on deviance explained. To evaluate the support for species-specific smoothers, we constructed equivalent models without interaction terms and compared nested models

based on AIC. For the 2023 data, we included a second parametric term of 'insecticide treatment' and a modified smoother allowing for an interaction between 'days in the field' and all four combinations of 'plant species' and 'insecticide treatment'.

We next fit a set of models to the individual presence and abundance data for the eight most abundant herbivores in both years. Due to the generally lower numbers of individual herbivores, GAMMs could not be consistently fit, thus we fit simpler Generalised Additive Models (GAMs, function 'gam in the *mgcv* package) for all herbivores. Data from the 2023 insecticide treatment was excluded from this analysis for simplicity. Herbivore abundance was analysed using negative binomial GAMs, while we specified a binomial family for presence-absence data. To avoid problems with excess zeros, we only included data from weeks in which an herbivore was observed more than once in the dataset, adjusting the number of nodes accordingly for each herbivore (Table S2).

2.3.2 Plant damage assessments

The amount of leaf damage in the 2022 field experiment was analysed by fitting a Generalized Linear Mixed-effects Model (GLMM) using the glmer function in the *lme4* package (Bates et al., 2015). We used the number of damaged and the number of undamaged leaves as a two-vector dependent variable, and this binomial variable was interpreted as the proportion of leaves with insect damage. The model included fixed factors of assessment week, plant species, and their interaction, while individual plant and observer were specified as random effects. To obtain p-values of the fixed effects, we used type III test implemented in the 'Anova' function from the *car* package (Fox & Weisberg, 2019). Post hoc pairwise comparisons (Tukey's HSD; $\alpha = 0.05$) were carried out using the *emmeans* package (Lenth, 2023).

The amount of leaf damage in the 2023 field experiment was analysed using a similar approach. We analysed the number of highly damaged leaves (leaves with more than 75% of area damaged) and the number of undamaged/low damage leaves as the two-vector dependent variable, and included factors of assessment week, plant species, insecticide treatment, and their interactions. Individual plant and observer were specified as random effects.

2.3.3 Plant growth

To make use of the unexpected drop in herbivore numbers following the early mowing of buffer strips in the 2022 field experiment, we quantified plant growth using repeated plant height measurements to identify changes in herbivore impact (supplementary methods). Biomass of plants harvested in weeks 8, 11, and 14 was analysed using a linear model (function 'gls' in the *nlme* package, Pinheiro, J. C. et al. 2021) of dry biomass as dependent variable and factors of plant species, harvest week, and their interaction as explanatory variables. Model fit was improved by allowing for separate variances for each harvest week (function 'varIdent' in the *nlme* package). Post hoc pairwise comparisons were carried out using *emmeans*.

2.3.4 Effects of herbivory and competition on plant biomass

We explicitly manipulated herbivore densities by insecticide applications in the 2023 field experiment. We analysed plant biomass of plants harvested after 10 weeks using a linear mixed-effects model (function 'lme' in the *nlme* package). Dry biomass was used as response variable and factors of plant species, insecticide treatment, competition treatment, and their interactions were fit as fixed effects. To account for the paired design of the competition treatment, we specified 'neighbourhood group' as a random effect. In the competition treatment, neighbourhood group refers to individual pots with pairs of plants, whereas in the individual treatment, neighbourhood group refers to three adjacent pots each with a single plant (one *B. nigra* plant randomly paired with two *E. cheiranthoides* neighbours). Model fit was improved by allowing for separate variances for each combination of plant species and insecticide treatment (function 'varIdent' in the *nlme* package). Post hoc pairwise comparisons for both models were carried out using *emmeans*.

3. Results

3.1 Herbivore community composition and phenology

We observed 14 different herbivores in 2022, with eight out of the nine most abundant herbivores occurring in substantially higher numbers on *B. nigra* than on *E. cheiranthoides* (Table S1). Total

herbivore abundance reached a peak in the fourth week, followed by a sharp decline (Figure 1a). This decline coincided with the removal of the buffer strips on each side of the experimental plot. We modelled herbivore abundance through time using a generalised additive mixed-effects model (GAMM) and found a strong main effect of plant species ($X^2 = 278.30$, df = 1, $p<0.001$) reflecting the overall difference in abundance. Model comparison revealed no support for a plant-specific relationship of total herbivore abundance through time (Table S2).

Individual herbivore species differed substantially in arrival and persistence time (Figure 1b), and even though herbivores generally were co-occurring, temporal dynamics significantly differed between plant species (Figures S2, S3). Using individual GAMs for presence and abundance data of the eight most abundant herbivores, we found significant main effects of plant species, support for plant-specific temporal relationships (i.e., plant-specific smoothers in GAMs), or both for all herbivores (Table S3). While results differed in magnitude and significance by herbivore, we generally found a higher likelihood of herbivore occurrence, higher herbivore abundance, and longer herbivore persistence on *B. nigra* compared to *E. cheiranthoides*. The largest exception to this pattern was the crucifer shield bug (adult *Eurydema ornata*, *E. oleracea*, and their larvae), which occurred at similar frequencies on both plants (Table S1), and at least during peak herbivore abundance, had a higher likelihood of occurrence on *E. cheiranthoides* (Figure S2).

We observed the same herbivores in 2023, except for three low-abundance species that were only observed in 2022. Again, seven out of the eight most abundant herbivores were less abundant on *E. cheiranthoides* (Table S1). As the buffer strips remained until the end of the experiment in this experiment, herbivores persisted for longer and we observed no sharp decline in total abundance (Figure 1c). Surprisingly, the insecticide treatment in 2023 did not reduce the total observed number of herbivores, but instead increased the observed number of aphids, pollen beetles, and flea beetles (Table S1). Modelling temporal dynamics in total herbivore abundance through time, we found strong support for main effects of plant species ($X^2 = 78.52$, df = 1, $p<0.001$) and insecticide treatment ($X^2 = 13.52$, df = 1, $p<0.001$), and model comparison revealed support for separate temporal relationships for each combination of plant species and insecticide treatment (Table S2).

Presence and abundance of individual herbivores also differed by plant species in 2023 (Figures S4, S5), with significant main effects of plant species, support for plant-specific temporal relationships, or both for all species (Table S3). Aphids and flea beetles in particular persisted for much longer in 2023, likely relying on the buffer strips for population maintenance. As in 2022, total abundance of crucifer shield bugs did not differ between *B. nigra* and *E. cheiranthoides* (Table S1), and we again found weak evidence for a higher likelihood of occurrence and higher abundance of this herbivore on *E. cheiranthoides* towards the end of the 2023 field experiment (Figures S4, S5).

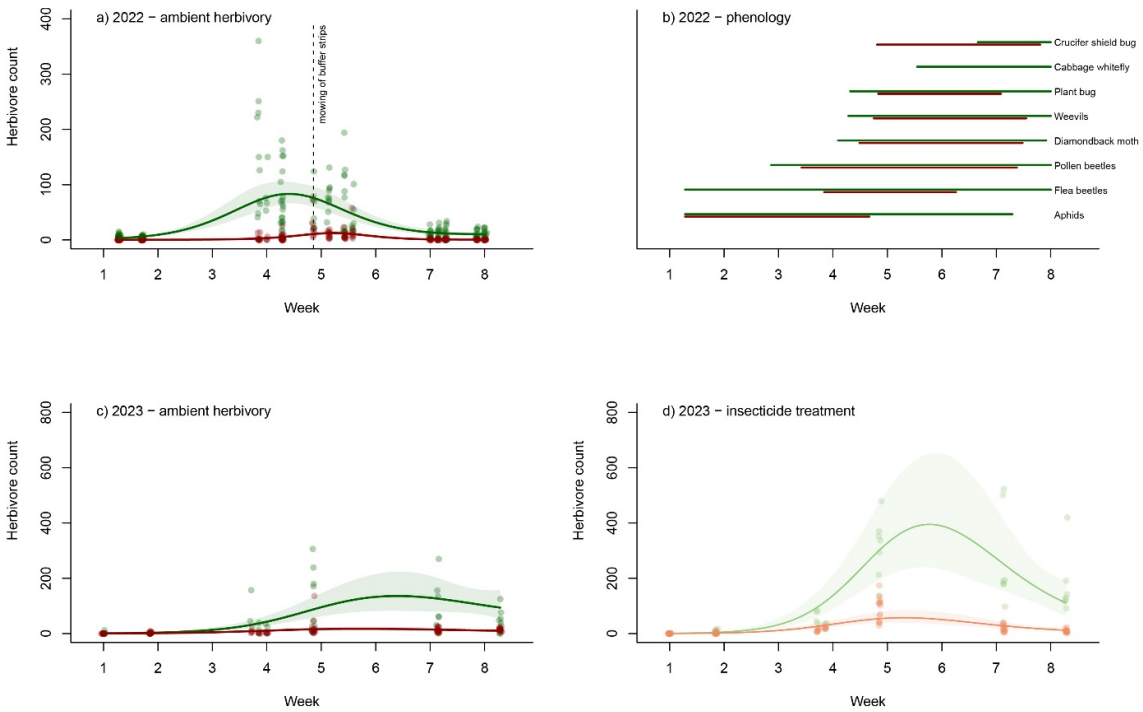

Figure 1. a) Total herbivore counts found on *B. nigra* (green) and *E. cheiranthoides* (red) in the 2022 field experiment. Points are observations on individual plants at a given time. Lines and shaded areas are the mean model predictions and 95% confidence intervals from a generalized additive mixed-effects model. The dashed line highlights the mowing of buffer strips next to the field experiment, which supported a high specialist herbivore density. b) Summarized phenology of the main herbivores observed in the 2022 field experiment. Lines connect the first and last day for which the predicted likelihood of herbivore presence (Figure S3) was > 0.1 (more than 10% of plants occupied). c) Total herbivore counts found on untreated plants ('ambient herbivory'), and d) total herbivore counts found on insecticide-treated plants in the 2023 field experiment. Lines

and symbols for plants in the insecticide treatment are distinguished by lighter colours. All models used ‘days in the field’ as temporal variable, but axes show ‘week’ for easier comparison to other results.

3.2 Plant damage assessments

Mirroring the results in herbivore abundance, leaf damage on *B. nigra* was substantially higher than on *E. cheiranthoides* in both years (Figure 2, Table S4). In the 2022 field experiment, damage to *B. nigra* was almost complete with virtually no leaf left undamaged, while less than 20% of leaves were damaged on *E. cheiranthoides* (Figure 2a). Damage slightly increased by week 6 for both plant species (weeks 3 vs. 6: z-ratio = -3.84, $p < 0.001$), before dropping considerably in week 9 (week 6 vs. 9: z-ratio = 13.82, $p < 0.001$).

In the 2023 field experiment, leaf damage on *B. nigra* plants exposed to ambient herbivory was again almost complete, thus, to avoid issues with zero variance in the smaller dataset, we analysed the proportion of heavily damaged leaves. Quantified damage was still substantially higher on *B. nigra* compared to *E. cheiranthoides* (Figure 2b, Table S4). Insecticide treatment strongly reduced damage on *E. cheiranthoides* plants in weeks 3 (ambient vs. sprayed: z-ratio = 4.73, $p < 0.001$) and 6 (ambient vs. sprayed: z-ratio = 3.21, $p = 0.001$), while on *B. nigra* an insecticide effect only was significant in week 9 (ambient vs. sprayed: z-ratio = 3.80, $p < 0.001$). Insecticide-treated *E. cheiranthoides* plants were slightly more damaged in week 9, but this effect was not significant (ambient vs. sprayed: z-ratio = -1.26, $p = 0.209$).

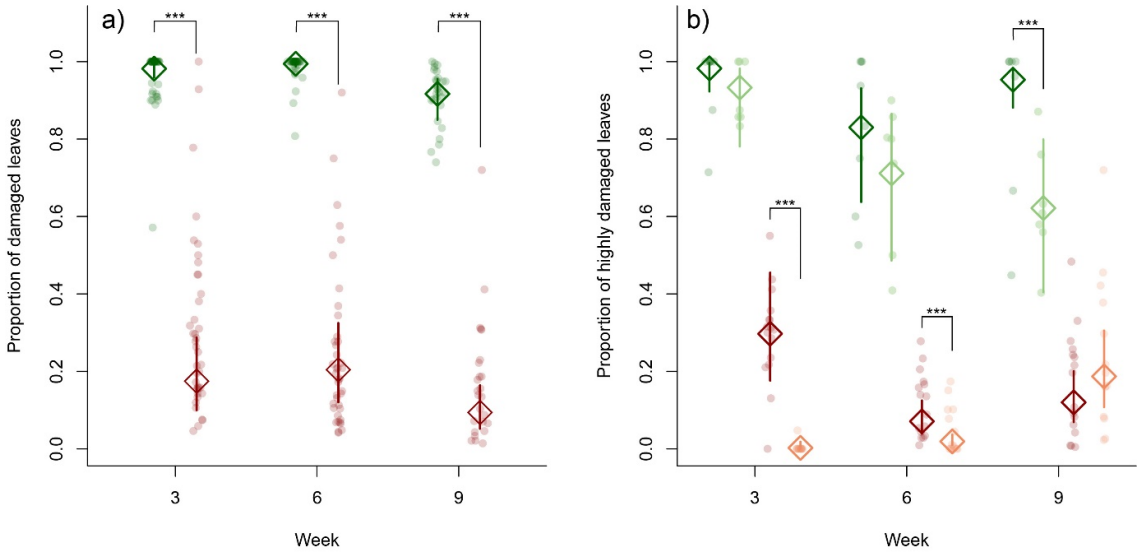

Figure 2. a) The proportion of damaged leaves on individual plants in weeks 3, 6, and 9 for *B. nigra* (green) and *E. cheiranthoides* (red) of the 2022 field experiment. Points are values for individual plants, while diamonds and lines are the means and 95% confidence intervals from a binomial GLMM. b) The proportion of highly damaged leaves (leaves with more than 75% area damaged) on individual plants in weeks 3, 6, and 9 for *B. nigra* and *E. cheiranthoides*. Plants under ambient conditions are shown in darker colours, while plants in the insecticide treatment are shown in lighter colours. Brackets with asterisks highlight significant linear contrasts for the pesticide treatment at the $p < 0.05$ level.

3.3 Plant nutritional traits

The substantially lower damage of *E. cheiranthoides* relative to *B. nigra* matches expectations for a well-defended plant, but naturally the two plant species differ in a multitude of other traits as well. For the 2022 field experiment, we therefore quantified several nutritional traits across the two species that might have affected herbivore preference independently of plant defence. Across three sampling points, *B. nigra* leaves contained significantly higher levels of soluble glucose and fructose (Fig. S6a-b), while *E. cheiranthoides* leaves contained higher levels of sucrose (Fig. S6c). Digestible starch content did not differ between species for most of the experiment, except for the final timepoint when starch content had dropped for both species, but more so for *E. cheiranthoides* (Fig. S6d). Similarly, total hydrolysable protein content only differed at later timepoints, with *B. nigra* containing up to 60% more protein per unit dry mass.

3.4 Plant growth and competition

To better understand herbivore impact on plants, we modelled plant growth in the 2022 field experiment using sequential measurements of stem height. We found strong differences between height growth of *B. nigra* and *E. cheiranthoides* (Figure 3a), with *E. cheiranthoides* reaching the inflection point of a logistic growth function after 29 days, while *B. nigra* reached this point only after 56 days (Table S5). During the first growth interval (weeks 3-6, high herbivore abundance), *B. nigra* plants in consequence only grew 0.31 (0.27-0.36, 95% confidence interval) cm day^{-1}, compared to *E. cheiranthoides* growing more than 5 times faster by this point (1.74 [1.53-1.93] cm day^{-1}). Growth of *B. nigra* only increased towards the end of the experiment when herbivore numbers decreased, and by the midpoint of the second growth interval (weeks 6-9, low herbivore abundance) the order was reversed, with *B. nigra* growing 1.30 (1.11-1.51) cm day^{-1} and *E. cheiranthoides* only growing 0.86 (0.75-0.95) cm day^{-1}.

The patterns in height growth of plants in the 2022 field experiment directly translated into differences in aboveground biomass (Figure 3b). In the subset of plants harvested after eight weeks, *B. nigra* had significantly lower biomass compared to *E. cheiranthoides* (pairwise linear contrast: $t = -4.33$, $df = 22$, $p < 0.001$). Mirroring the increased height growth of *B. nigra*, these differences had disappeared by week 11 ($t = -0.43$, $df = 21$, $p = 0.676$) and 14 ($t = -0.019$, $df = 17$, $p = 0.985$).

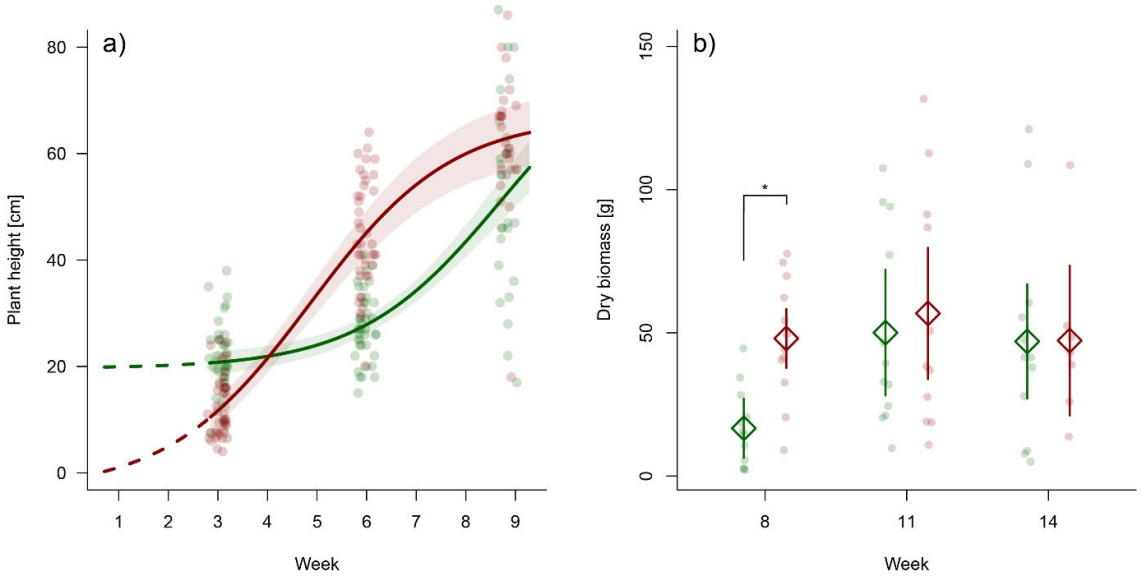

Figure 3. a) Height growth over nine weeks for *B. nigra* (green) and *E. cheiranthoides* (red) in the 2022 field experiment. Points are individual plant values and solid lines are model fits from a logistic regression. Shaded areas are the 95% confidence intervals (population prediction intervals). Plant height was first recorded in the third week, resulting in greater uncertainty for the initial growth period (dashed lines). b) Total aboveground biomass of subsets of plants harvested after 8, 11, and 14 weeks in the 2022 field experiment. Diamonds and lines are means and 95% confidence intervals from a linear model. The bracket denotes a significant pairwise linear contrasts at the $p < 0.05$ level.

Based on the strong impacts of herbivores on plant biomass we observed in 2022, we explicitly manipulated herbivore pressure and competitive environment in the 2023 field experiment. For single plants under ambient herbivore pressure, biomass of *B. nigra* plants after 10 weeks of growth was close to zero, while *E. cheiranthoides* plants could grow seemingly unimpaired (Figure 4a). Even though application of insecticide did not remove herbivores (Figure 1c-d) and only partially reduced observed damage on *B. nigra* (Figure 2b), insecticide-treated *B. nigra* plants grew better and reached a higher biomass (pairwise linear contrast: $t = -3.37$, $df = 80$, $p = 0.006$). In comparison, *E. cheiranthoides* gained no equivalent benefit from insecticide application ($t = -1.05$, $df = 80$, $p = 0.723$).

Growing in intense competition with *E. cheiranthoides* had little effect on *B. nigra* biomass, which again suffered heavily under ambient herbivory, but benefitted from insecticide applications

(Figure 4b). Biomass of *E. cheiranthoides* growing in competition with *B. nigra* under ambient herbivory was likewise largely unchanged, but application of insecticide substantially reduced *E. cheiranthoides* biomass (t = 2.72, df = 80, p = 0.039), likely as a direct result of increased competition from less-attacked *B. nigra* plants. A reduction in biomass has fitness consequences for an annual plant such as *E. cheiranthoides*, with aboveground biomass directly relating to seed production in this species (Figure S7).

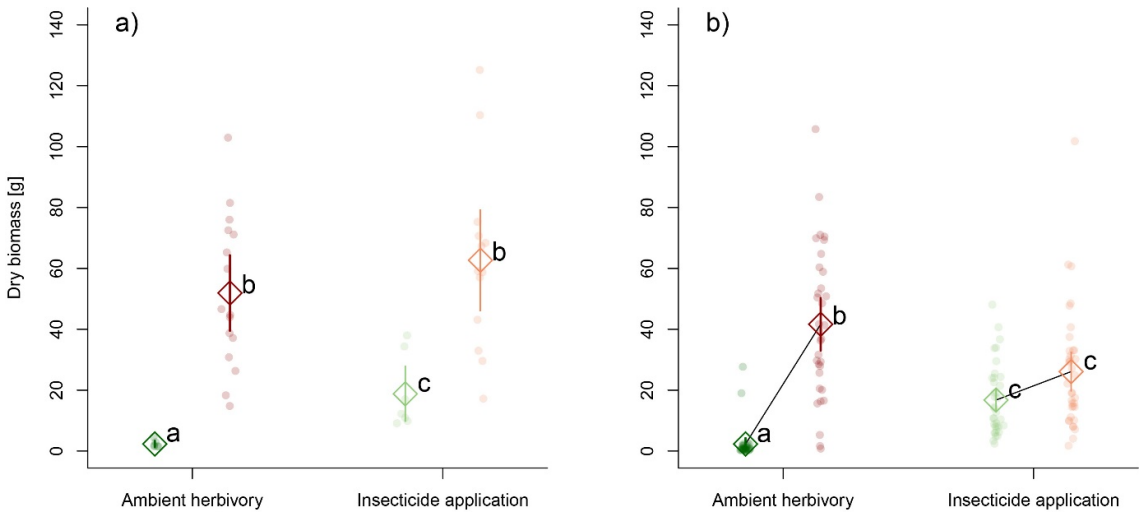

Figure 4. a) Biomass of individual *B. nigra* (green) and *E. cheiranthoides* (red) plants harvested after 10 weeks of growth in the 2023 field experiment. Plants were growing individually in buried pots, separated from the nearest neighbour by 0.5 m in all directions. Points are individual plant values, while diamond symbols and lines are means and 95% confidence intervals from a linear model. Plants in the insecticide treatment are distinguished by lighter colours. b) Biomass of paired plants growing inside the same buried pot, experiencing intense competition above- and belowground. Diamond symbols and lines are means and 95% confidence intervals from a linear mixed effects model. Letters denote significant linear contrasts at the p<0.05 level.

4. Discussion

The dually defended *E. cheiranthoides* was attacked by substantially fewer herbivores than its Brassicaceae relative *B. nigra* in two years of field observations. Herbivores caused severe damage to leaves of *B. nigra*, and in consequence, growth of *B. nigra* was almost completely suppressed by herbivory when insect herbivores were abundant. In contrast, even during peak abundance, the limited

herbivore damage on *E. cheiranthoides* had no impact on its growth, matching a successful escape from herbivore-imposed selection. In fact, experimental manipulation of herbivore abundances in the second year revealed not only a lack of negative impact of herbivore selection, but a benefit of herbivory on *E. cheiranthoides* under conditions of intense competition with *B. nigra*. Our study thus reveals the dually defended *E. cheiranthoides* to be both highly resistant and tolerant to herbivore damage, but this defensive superiority is paired with weak competitive ability.

Herbivores attacking *B. nigra* and *E. cheiranthoides* were mostly Brassicaceae specialists with known tolerance of the GSL defence. These herbivores were likely present in high numbers in the surrounding Brassicacaea crops and 'spilled over' onto experimental plants, explaining the sharp decline in herbivore numbers on experimental plants when cover strips were mowed prematurely in 2022. Despite these somewhat unusual conditions, other field studies commonly find specialist insects to be the dominant herbivores of Brassicaceae plants (Bidart-Bouzat & Kliebenstein, 2008; Mertens et al., 2021), likely because the GSL defence acts as potent filter against many generalist herbivores (but see Zalucki et al., 2021). Among the remaining specialist herbivores in our field surveys, most showed a strong decrease in total abundances on *E. cheiranthoides*, but none exhibited complete avoidance of the dually defended plant. Importantly, we did not distinguish between perching and feeding herbivores in our study, and several of the observed herbivores are highly mobile (e.g., pollen beetles, flea beetles). It is thus feasible that herbivores were constantly arriving on *E. cheiranthoides*, but after initial sampling decided to leave the plant again. A similar effect may explain the higher numbers of observed herbivores on insecticide-treated plants in 2023, where insecticide application reduced plant damage and likely increased plant quality, which temporarily may have attracted higher numbers of mobile herbivores.

Although the comparison of *B. nigra* and *E. cheiranthoides* in our study highlights differences in herbivore-imposed selection pressures experienced by these plants, it can only provide partial insights into a causal role of cardenolides in plant defence. Many other traits besides cardenolides vary between the two plant species, as for example our measurements of foliar nutrients revealed *B. nigra* to be of higher nutritional quality than *E. cheiranthoides*. Even though the large differences in herbivore preference were unlikely caused by these relatively modest differences in nutritional

quality, other unmeasured traits may have been driving herbivore preference and feeding behaviour. In a recent study, Younkin et al. (2023) used a newly developed CRISPR/Cas9 knockout line of *E. cheiranthoides* with abolished cardenolide production to more directly evaluate the defensive role of cardenolides in the laboratory and field. In their study, a few specialist herbivores that never attacked wildtype plants were able to feed on knockouts, while other herbivores either showed increased feeding on knockouts relative to wildtype, or showed no discrimination between plants at all (Younkin et al., 2023). These findings suggest a more nuanced role of cardenolides in the defence of *E. cheiranthoides*, with the novel compounds acting as highly effective qualitative defences against some herbivores, and as less effective quantitative defences – or even ineffective compounds – against others. To explore the full role of cardenolide variation in herbivore damage, an in-depth analysis of intraspecific variation among natural *E. cheiranthoides* genotypes may prove useful.

Across both years of our field experiment, we observed a suite of herbivores that is representative of the main Brassicaceae herbivores in central Europe (Mertens et al., 2021). Interestingly, we never observed larvae of the cabbage white butterfly (*Pieris rapae*) feed on experimental plants despite a high adult abundance in the field. The larvae of *P. rapae* are major herbivores of many Brassicaceae plants but they are highly susceptible to the cardenolides in *E. cheiranthoides* (Sachdev-Gupta et al., 1993) and respond positively to cardenolide knockout lines (Younkin et al., 2023). While the lack of *P. rapae* larvae on *E. cheiranthoides* is thus expected, their absence on *B. nigra* is more surprising, given that adults of *P. rapae* were commonly observed throughout both field seasons. It thus possible that *B. nigra* may have gained associational resistance (Atsatt & O'dowd, 1976) against *P. rapae* from their *Erysimum* neighbours.

Specialist herbivores of the Brassicaceae plants commonly have low specialization among different Brassicaceae species (Mertens et al., 2021). Of the herbivores we observed, most consistently preferred *B. nigra* over *E. cheiranthoides*, regardless of taxonomic group or feeding guild (e.g., caterpillars, flea beetles, aphids). The only exceptions to this pattern were the crucifer shield bugs (*Eurydema ornata*, *E. oleracea*, and their larvae), which occurred on both experimental plants at equal abundances, and at least over select periods, were more likely to occur on *E. cheiranthoides*. Several species of *Eurydema* are known to feed on different *Erysimum* species (Gómez, 2005), and

both *E. ornata* and *E. oleracea* are commonly found feeding on natural *E. cheiranthoides* plants (personal observation). Despite this apparently frequent association, *Eurydema* bugs remain broadly generalised herbivores of many Brassicaeae species (Piersanti et al., 2020). However, *Eurydema* bugs show clear preferences for the volatile profile of certain Brassicaceae host plants (Piersanti et al., 2020), but it is currently unclear whether *Eurydema* bugs favour *Erysimum* for its nutritional quality, its defensive arsenal, or its defensive profile, and whether *Eurydema* is in the process of becoming more intimately associated with this host plant.

Damage by herbivores can reduce plant growth directly by reducing photosynthetically active leaf area and removing limiting resources, or indirectly by inducing plant responses that divert resources away from growth to defence (Attaran et al., 2014; Zangerl et al., 2002). Plant species differ in their relative allocation of resources to herbivore resistance and competitive ability, and these functions commonly trade off (Agrawal, 2020). In consequence, herbivores can have substantial effects on the composition of entire plant communities, i.e., by preferentially attacking competitively superior susceptible host plants, thereby allowing for competitively inferior resistant plants to increase in frequency (Crawley, 1989; Myers & Sarfraz, 2017).

Trade-offs between herbivore resistance and competitive ability or other primary functions are common across the plant kingdom (Züst & Agrawal, 2017). Resistance traits such as chemical defences often represent large investments of both energy and other resources for a plant, and GSL production alone has been estimated to consume 15% of a plant's total photosynthetic energy (Bekaert et al., 2012). Even though such metabolic costs are not always apparent and may sometimes be masked in species comparisons by unrelated trait variation (Koricheva et al., 2004), comparisons of plants that differ primarily in defence traits of interest tend to reveal such costs (Züst et al., 2011). While *B. nigra* and *E. cheiranthoides* differ in a diversity of traits, they both share the same molecular machinery for GSL production, while *E. cheiranthoides* additionally maintains the machinery for cardenolide production which has been shown to trade off with growth in another plant system (Züst et al., 2015). It is therefore more than likely that the unusual dual investment by *E. cheiranthoides* is the primary cause for the weak competitive ability demonstrated in this study.

Despite the weak competitive ability of *E. cheiranthoides* (and likely that of its close relatives), the genus *Erysimum* has undergone a recent radiation that coincided with the gain of cardenolides (Züst et al., 2020), suggesting that this key innovation facilitated *Erysimum* to escape specialized herbivores and diversify in enemy-free space. However, both *E. cheiranthoides*, and species in the genus more generally, mostly occupy marginal habitats that are often devoid of vegetation, rarely forming large populations. *Erysimum* plants thus appear to lack the ability to become dominant species within a community, despite being better defended than most of their heterospecific neighbours. Being locally rare may in fact work in *Erysimum*'s favour, as a rare hosts may be less likely to select for the types of specialized resistance that are common in other cardenolide-producing plants (Dobler et al., 2012; Petschenka et al., 2017).

In conclusion, the gain of a novel defence appears to have allowed *Erysimum* plants to escape herbivore-imposed selection, if not herbivory entirely. However, the costs required to maintain this 'enemy-free space' are likely so substantial that plants can only thrive under conditions where heterospecific competitors are suppressed by strong herbivory. As such conditions are rare, *Erysimum* is currently relegated to survive in marginal habitats, yet changing environmental conditions might well favour this unusually well-defended plant in the future.

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Author contributions

T.Z. conceived the study; K.W. and T.Z. designed the experiments; all authors collected and analysed the data; K.W. and T.Z. led the writing of the manuscript. All authors contributed critically to the drafts and gave final approval for publication.

Data availability statement

Data from this paper can be accessed through Dryad.

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Supplementary Materials

Supplementary Methods

1. *Plant growth*

Using plants heights measured in weeks 3, 6, and 9, we modelled height growth of the plant species using a 4-parameter logistic function of the form

$$H = H_0 + \frac{K - H_0}{1 + e^{\left(-r\left(t - t_{mid}\right)\right)}} \qquad \text{eqn 1,}$$

where the height H at time t is a function of the initial plant height H_0 (i.e., height at transplanting), an asymptotic height K, a growth rate r, and the time t_{mid} at which the inflection point is reached (i.e., height halfway between H_0 and K). We fitted eqn. 1 to plant height using the *nlme* function in the nlme package, treating plant identity as random effects to account for repeated measures of the same individual, and fitting fixed effects of plant species on the different growth parameters. As we only recorded height three times, we were limited to estimating two parameters. We therefore manually set parameters t_{mid} and r to species-specific values in an iterative process. For each species separately, we first set parameter r to 0.1 and fit models for a range of t_{mid} values (20-60 days). We compared the resulting models using AIC and selected the optimal species-specific value of t_{mid}, before repeating the procedure for a range of r values (0.05-0.5). With both parameters set to optimal species-specific values, we then analysed the full data to estimate the remaining two parameters.

To compare growth between the two species at different points in time, we estimated the absolute growth rate (AGR) using the function

$$AGR = \frac{r(K - H_0)e^{\left(-r\left(t - t_{mid}\right)\right)}}{\left(1 + e^{\left(-r\left(t - t_{mid}\right)\right)}\right)^2} \qquad \text{eqn 2.}$$

Using equation 2, we estimated AGR for the midpoints of the first (weeks 3-6) and second growth interval (weeks 6-9). For each set of AGR values, we generated 95% population prediction intervals by drawing random values from the sampling distribution of each of the two estimated parameters (Bolker, 2008).

2. *Leaf traits*

Leaves from subsets of plants were harvested in weeks 4, 7, and 10 (one week after damage assessments) to determine changes in nutritional quality of plants over time. Each plant was only sampled once, with plants from blocks 1-3 sampled in week 4, plants from blocks 4-6 sampled in week 7, and plants from block 7-10 sampled in week 10. For each sampling, we removed 5-10 leaves from *B. nigra*, or 10-20 leaves from *E. cheiranthoides*, selecting leaves from the top, middle, and bottom part of each plant. Leaves from the same plant were wrapped together in aluminium foil and immediately placed on dry ice. Frozen leaf samples were then transported back to the laboratory and stored at -80 °C, before freeze-drying under vacuum for 48 h. We homogenized dried leaves for each plant and

prepared two replicate samples weighing 10 mg each in 2 mL screw-cap plastic tubes (Avantor, USA). Replicate samples were used for determination of total protein content and determination of sugar and starch content.

2.1 Total protein content

The total protein content of leaves was determined using the protocol of Clissold et al. (2006). Briefly, 10 mg of ground leaf material was extracted twice using a total volume of 0.8 mL 0.1M NaOH. For extraction, samples were sonicated for 30 minutes and heated at 90 °C for 15 min, followed by centrifugation. Supernatants were pooled and neutralized using 6M HCl. We then precipitated proteins by adding 90 µL trichloroacetic acid solution (100% w/v, Sigma-Aldrich, Switzerland), followed by centrifugation and washing of the protein pellet with cold acetone. We re-suspended dried pellets using 1 mL of 0.1M NaOH, diluted the dissolved protein 1:20 with water, and assayed 50 µL of dilute protein solution with 200 µL QuickStart™ Bradford Dye (BioRad, Switzerland), reading absorbance at 595 nm on a microplate reader. Each sample was assayed in triplicate and protein concentrations were calculated using a concentration curve of Immunoglobuline G (Sigma-Aldrich, Switzerland; 0, 1, 2, 4, 6, 8, 12 µg protein per assay).

2.2 Soluble sugars and starch content

Sugar content was determined using the protocol of Machado et al. (2013). Ground plant material was extracted with 0.5 mL 80% ethanol in water, and re-extracted twice using 0.5 mL 80% ethanol in water. For each extraction, samples were vortexed and heated at 78 °C for 15 min, followed by centrifugation. Supernatants were pooled, and 25 µL of pooled extracts were used for determination of glucose, fructose, and sucrose using an enzymatic assay as described by Velterop & Vos (2001). Briefly, glucose was converted to glucose-6-phosphate by hexokinase (Roche, Switzerland) in the presence of ATP (Sigma-Aldrich, Switzerland), and further reacted to 6-phosphogluconate by glucose-6-P-dehydrogenase (Roche, Switzerland) in the presence of $NADP^+$ (Sigma-Aldrich, Switzerland). The latter reaction releases NADPH, which was quantified photometrically at 340 nm on a microplate reader. Reactions were carried out in 50 mM HEPES buffer (pH 7, with 5 mM $MgCl_2$). After readings stabilized, we added phosphoglucoisomerase (Roche, Switzerland) to convert fructose to glucose, and finally we added invertase (Sigma-Aldrich, Switzerland) to convert sucrose into glucose and fructose. Each sample was run in triplicate, and sugars were quantified relative to a concentration curve of glucose (25 µL of 0, 0.125, 0.25, 0.5, 1, and 2 mM solutions).

We also determined starch content in the plant material remaining after sugar extraction using the protocol of Smith & Zeeman (2006). We added 1 mL of water to each plant precipitate, and diluted 100 µL of this suspension with 500 µL water. Dilute suspensions were autoclaved for 1 h and left to cool down to 37 °C. We then added 500 µL of an enzyme solution consisting of 50 mM sodium-acetate buffer (pH 5.5), amyloglucosidase (Roche, Switzerland) and α-amylase (Sigma-Aldrich, Switzerland).

Solutions were incubated at 37 °C overnight, and finally we quantified sugars in 25 µL of supernatant using the same enzymatic assay as above.

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Table S1. Mean (± 1 SE) numbers of herbivores per plant found sitting or feeding on *B. nigra* and *E. cheiranthoides*. Numbers are the cumulative per-plant counts of six observations, averaged across all plants of the same species and treatment. Flea beetles, large flea beetles, weevils, and leaf hoppers are classes of taxonomically complex species for which field determination was deemed impractical. Bold values highlight significantly different numbers between plant species in a specific year and treatment ($p < 0.05$, generalized linear model of cumulative counts using a negative binomial error structure).

				2022		2023		2023	
				ambient herbivory		ambient herbivory		insecticide treatment	
Herbivore	Scientific name	Order	Stage	*B. nigra*	*E. cheiranthoides*	*B. nigra*	*E. cheiranthoides*	*B. nigra*	*E. cheiranthoides*
				n = 40	n = 40	n = 8	n = 16	n = 8	n = 16
flea beetle		Coleoptera	adult	**24.70 ± 5.29**	**1.52 ± 0.35**	**85.5 ± 27.43**	**17.06 ± 2.23**	**181.00 ± 38.47**	**3.36 ± 0.63**
pollen beetle	*Meligethes aeneus*	Coleoptera	adult	**108.20 ± 12.84**	**12.10 ± 2.41**	**17.12 ± 6.56**	**9.38 ± 1.23**	**102.29 ± 21.72**	**11.79 ± 1.64**
weevil		Coleoptera	adult	**4.03 ± 0.52**	**1.07 ± 0.24**	**7.75 ± 1.5**	**1.31 ± 0.25**	**27.86 ± 4.76**	**2.5 ± 0.68**
leaf miner	*Phytomyza sp.*	Diptera	juvenile	**0.40 ± 0.16**	**0.07 ± 0.06**	0	0.12 ± 0.12	0	0
aphid		Hemiptera	adult/juvenile	**20.32 ± 6.67**	**0.82 ± 0.19**	**119 ± 38.03**	**19.38 ± 6.51**	**431.57 ± 81.78**	**116.29 ± 12.9**
cabbage whitefly	*Aleyrodes sp.*	Hemiptera	adult	**6.78 ± 1.46**	**0.17 ± 0.12**	0.25 ± 0.25	0.38 ± 0.22	**11.43 ± 7.75**	**0.57 ± 0.25**
leaf hopper		Hemiptera	adult	0.10 ± 0.05	0.07 ± 0.04	0	0	0	0
crucifer shield bug	*Eurydema sp.*	Heteroptera	adult/juvenile	0.55 ± 0.22	1.07 ± 0.25	1.25 ± 0.37	1.31 ± 0.37	0.29 ± 0.29	0.93 ± 0.34
plant bug	*Lygus sp.*	Heteroptera	adult/juvenile	**4.55 ± 0.71**	**0.48 ± 0.14**	**1.62 ± 0.84**	**0.06 ± 0.06**	**2.14 ± 0.46**	**0.14 ± 0.1**
plant bug	*Closterotomus sp.*	Heteroptera	adult	0.12 ± 0.06	0.03 ± 0.02	**1.00 ± 0.50**	**0.06 ± 0.06**	**1.14 ± 0.51**	**0.14 ± 0.1**
cabbage looper	*Trichoplusia ni*	Lepidoptera	juvenile	0	0.03 ± 0.02	0	0	0	0
cabbage moth	*Mamestra brassicae*	Lepidoptera	juvenile	0.03 ± 0.02	0	0	0	0	0
diamondback moth	*Plutella xylostella*	Lepidoptera	adult/juvenile	**2.35 ± 0.38**	**0.75 ± 0.16**	0.12 ± 0.12	0.31 ± 0.15	**4 ± 1.02**	**0.43 ± 0.17**

Table S2. Model comparison of generalized additive mixed-effects models (GAMMs) of the temporal dynamics in total herbivore abundance in two years. For 2022, AIC indicated better support for a model lacking a species-specific smoother. For 2023, AIC supported a model with separate smoothers for each combination of plant species and insecticide treatment ('PxT').

Year	Model parameters	Df	AIC
2022	~ Plant + s(Day, by=Plant)	8	1441.42
	~ Plant + s(Day)	**6**	**1419.81**
2023	**~ Plant + Treatment + s(Day, by=PxT)**	**13**	**931.21**
	~ Plant + Treatment + s(Day, by=Plant)	9	937.59
	~ Plant + Treatment + s(Day, by=Treatment)	9	937.18
	~ Plant + Treatment + s(Day)	7	934.17

Table S3. Test statistics for generalized additive models (GAMs) of the temporal dynamics in presence and abundance of the most abundant herbivores in 2022 and 2023. For each herbivore and year, a separate binomial GAM and negative-binomial GAM was fit to presence and abundance data, respectively. For each model, a parametric term of 'plant species' (main effect) and a plant-specific smoother was specified. Significance of the main effect was directly assessed by percentage deviance explained (Type III Wald chisquare tests). Support for plant-specific smoothers was assessed by comparing each full model to a nested single-smoother model using AIC. A plant-specific smoother was considered necessary to explain temporal trends in the data if the full model had a lower AIC by >2 units. The number of nodes in each smoother (parameter k in GAM) were adjusted according to the number of weeks in which herbivores were observed. For 2023, data of the insecticide treatment was excluded from the analysis. Two herbivores present in 2022 had insufficient data in 2023 to fit GAMs.

	2022					2023 (ambient herbivores only)				
	Herbivore presence		Herbivore abundance			Herbivore presence		Herbivore abundance		
Herbivore	main effect plant species	plant-specific smoother	main effect plant species	plant-specific smoother	k	main effect plant species	plant-specific smoother	main effect plant species	plant-specific smoother	k
aphids	**df = 1, X^2 = 32.46, p < 0.001**	ΔAIC: 2.46	**df = 1, X^2 = 43.94, p < 0.001**	**ΔAIC: -8.06**	5	df = 1, X^2 = 0.21, p = 0.650	ΔAIC: 2.25	df = 1, X^2 = 1.59, p = 0.2075	**ΔAIC: -11.64**	4
cabbage whitefly	**df = 1, X^2 = 20.80, p < 0.001**	ΔAIC: 1.88	**df = 1, X^2 = 44.54, p < 0.001**	ΔAIC: 1.86	3	n/a	n/a	n/a	n/a	n/a
crucifer shield bug	df = 1, X^2 = 0.16, p = 0.693	**ΔAIC: -3.88**	df = 1, X^2 = 2.29, p = 0.130	ΔAIC: 0.6	3	df = 1, X^2 = 0.87, p = 0.352	**ΔAIC: -3.64**	df = 1, X^2 = 1.59, p = 0.2071	**ΔAIC: -6.55**	3
diamondback moth	**df = 1, X^2 = 8.85, p = 0.003**	ΔAIC: 2.13	**df = 1, X^2 = 18.15, p < 0.001**	ΔAIC: 3.1	3	n/a	n/a	n/a	n/a	n/a
flea beetle	**df = 1, X^2 = 7.10, p = 0.008**	**ΔAIC: -12.58**	**df = 1, X^2 = 11.43, p < 0.001**	**ΔAIC: -20.55**	5	**df = 1, X^2 = 4.40, p = 0.036**	**ΔAIC: -3.31**	**df = 1, X^2 = 73.06, p < 0.001**	ΔAIC: 5.28	5
plant bug	**df = 1, X^2 = 28.88, p < 0.001**	**ΔAIC: -5.02**	**df = 1, X^2 = 29.91, p < 0.001**	**ΔAIC: -9.88**	3	**df = 1, X^2 = 7.72, p = 0.006**	ΔAIC: -0.49	**df = 1, X^2 = 8.44, p = 0.004**	ΔAIC: -1.53	3
pollen beetle	df = 1, X^2 = 0.99, p = 0.319	**ΔAIC: -3.55**	**df = 1, X^2 = 17.50, p < 0.001**	**ΔAIC: -22.14**	4	df = 1, X^2 = 1.41, p = 0.234	**ΔAIC: -19.93**	df = 1, X^2 = 0.11, p = 0.736	**ΔAIC: -31.88**	3
weevil	**df = 1, X^2 = 23.35, p < 0.001**	ΔAIC: -1.5	**df = 1, X^2 = 11.49, p < 0.001**	**ΔAIC: -6.39**	3	**df = 1, X^2 = 5.08, p = 0.024**	ΔAIC: 2.97	**df = 1, X^2 = 26.01, p < 0.001**	ΔAIC: 3.94	3

Table S4. Models for the proportion of damaged leaves (related to Figure 2). a) Model structure for the GLMM used to analyse the 2022 field data, and b) corresponding model statistics. P-values for the fixed effects were obtained using the 'Anova' function from the car package. c) Model structure for the GLM used to analyse the 2023 field data, and d) corresponding model statistics.

a) GLMM: Proportion of damaged leaves: cbind(damaged,undamaged)

~ Week * Plant species + (1|PlantID) + (1|ObserverID)

b) Analysis of Deviance Table (Type III Wald Chisquare tests)

	Chisq	Df	(Pr > ChiSq)
(intercept)	88.242	1	< 0.001
Week	140.500	2	< 0.001
Plant species	241.374	1	< 0.001
Week:Plant species	61.521	2	< 0.001

c) GLMM: Proportion of highly damaged leaves: cbind(highly damaged,(low+undamaged))

~ Week * Plant species * Insecticide treatment + (1|PlantID) + (1|ObserverID)

d) Analysis of Deviance Table (Type III Wald Chisquare tests)

	Chisq	Df	(Pr > ChiSq)
Week	20.04	2	< 0.001
Plant species	34.04	1	< 0.001
Insecticide treatment	1.83	1	0.177
Week:Plant species	5.54	2	0.063
Week:Insecticide treatment	21.11	2	< 0.001
Plant species:Insecticide treatment	6.33	1	0.012
Week:Plant species:Insecticide treatment	98.32	2	< 0.001

Table S5. Parameters of the 4-parameter logistic model of plant height growth. As height was only measured three times, only two parameters could be estimated. Therefore, the parameters t_{mid} and r were set to biologically relevant values and were then further optimized by repeatedly running separate models for *B. nigra* and *E. cheiranthoides* across a range of values of t_{mid} and r. Parameters resulting in the lowest model AIC were selected for each species. With t_{mid} and r set, parameters H_0 and K were then estimated using an *nlme* model of the full data. Parameters are thus either model estimates (±1 SE) or single optimized values. Note that the negative estimate of H_0 for *E. cheiranthoides* (corresponding to negative plant height at time of transplanting) is likely due to limited data on early development.

	B. nigra	*E. cheiranthoides*
H_0 (estimated)	19.66 ± 1.08	-3.40 ± 1.61
K (estimated)	82.70 ± 4.55	66.98 ± 3.40
t_{mid} (set)	56	29
r (set)	0.1	0.1

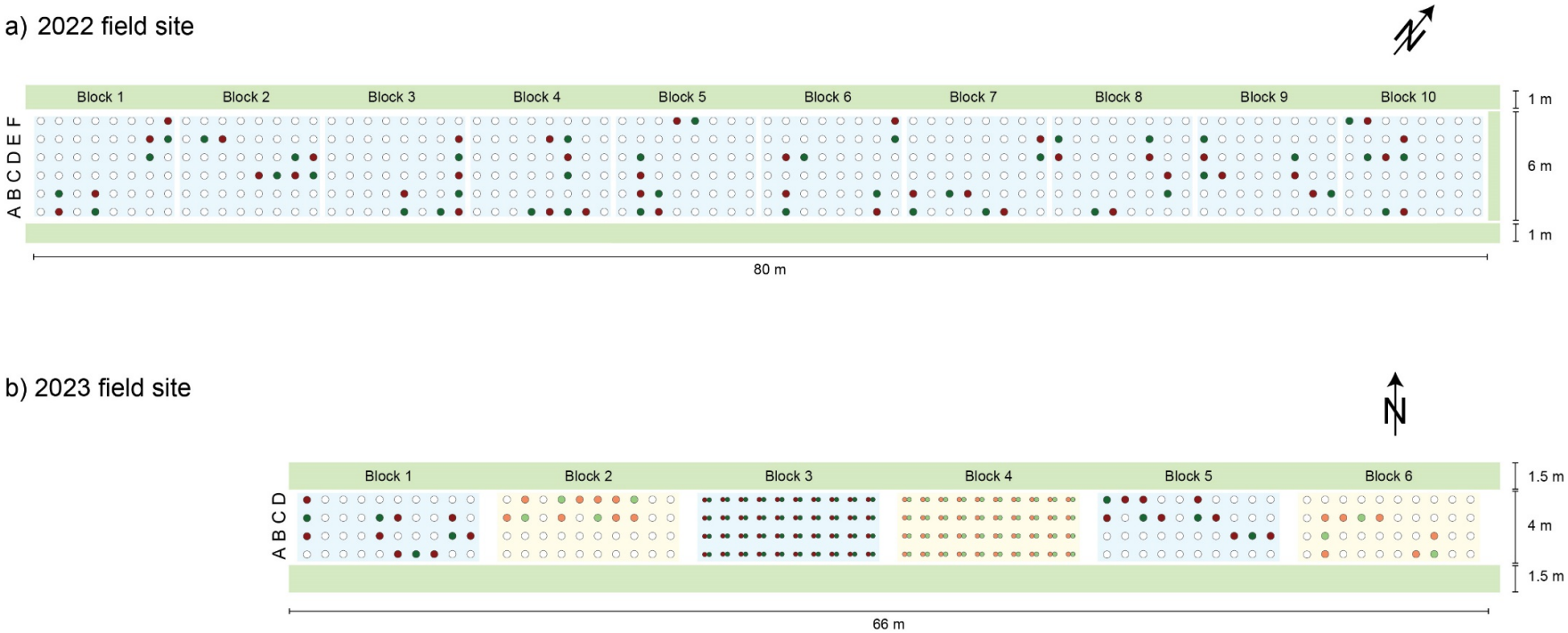

Figure S1. a) Schematic of the 2022 field site. Shown are the ten replicate blocks (blue squares), with focal *B. nigra* plants (green circles) and one matched randomly selected *E. cheiranthoides* neighbour (red circles) highlighted. Empty circles are additional *E. cheiranthoides* plants not included for the analyses of this study. b) Schematic of the 2023 field site. Shown are the three blocks under ambient herbivory (blue squares), and the three blocks receiving weekly applications of insecticide (yellow squares). Focal *B. nigra* plants (green circles) and two matched randomly selected *E. cheiranthoides* neighbour (red circles) used for this study are highlighted. Plants in the insecticide treatment are shown in lighter colours, corresponding to the colour scheme in the main figures. Paired circles in blocks 3 and 4 represent pairs of *B. nigra* and *E. cheiranthoides* plants growing closely together in the same buried pot to impose plant-plant competition. Buffer strips sown with a mixture of *Raphanus sativus* and *Avena strigosa* are shown as light green rectangles for both years. Both sites were located immediately adjacent to oilseed rape crop fields (not shown).

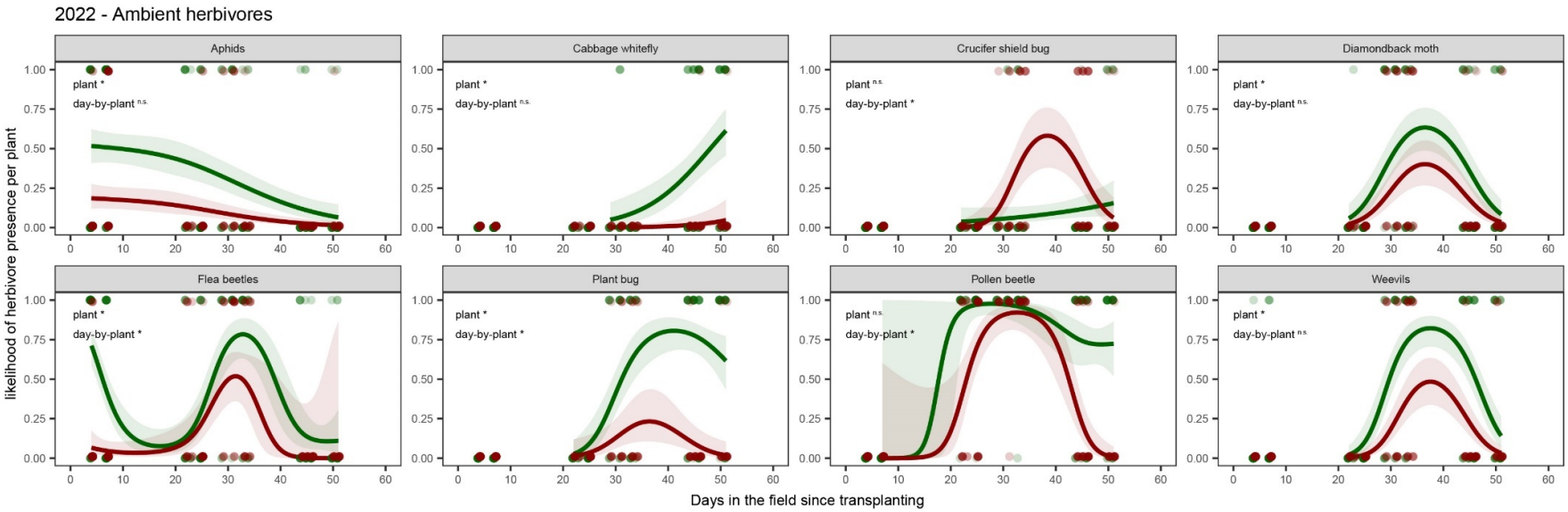

Figure S2. Likelihood of herbivore presence per plant over 9 weeks for *B. nigra* (green) and *E. cheiranthoides* (red) in the 2022 field experiment. The X-axis shows the days since plants were placed in the field, and the y-axis shows the likelihood of herbivore presence per plant. Points are observations on individual plants and lines are model fits from generalized additive models (GAMs). Shaded areas are the 95% confidence intervals. In each panel, 'plant' refers to statistical support of a main effect of plant species, and 'day-by-plant' for support of a species-specific smoother. See Table S3 for details.

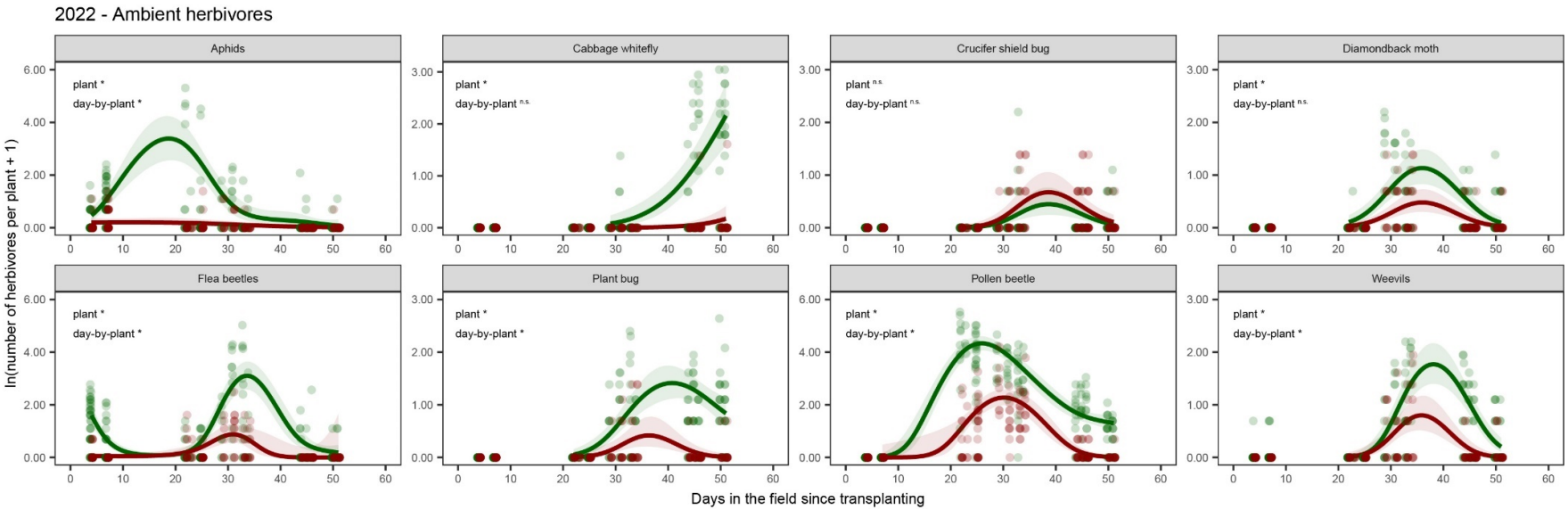

Figure S3. Number of herbivores per plant over 9 weeks for *B. nigra* (green) and *E. cheiranthoides* (red) in the 2022 field experiment. The X-axis shows the days since plants were placed in the field, and the y-axis shows the natural logarithm of the number of herbivores per plant. Points are observations on individual plants and lines are model fits from generalized additive models (GAMs). Shaded areas are the 95% confidence intervals. In each panel, 'plant' refers to statistical support of a main effect of plant species, and 'day-by-plant' for support of a species-specific smoother. See Table S3 for details.

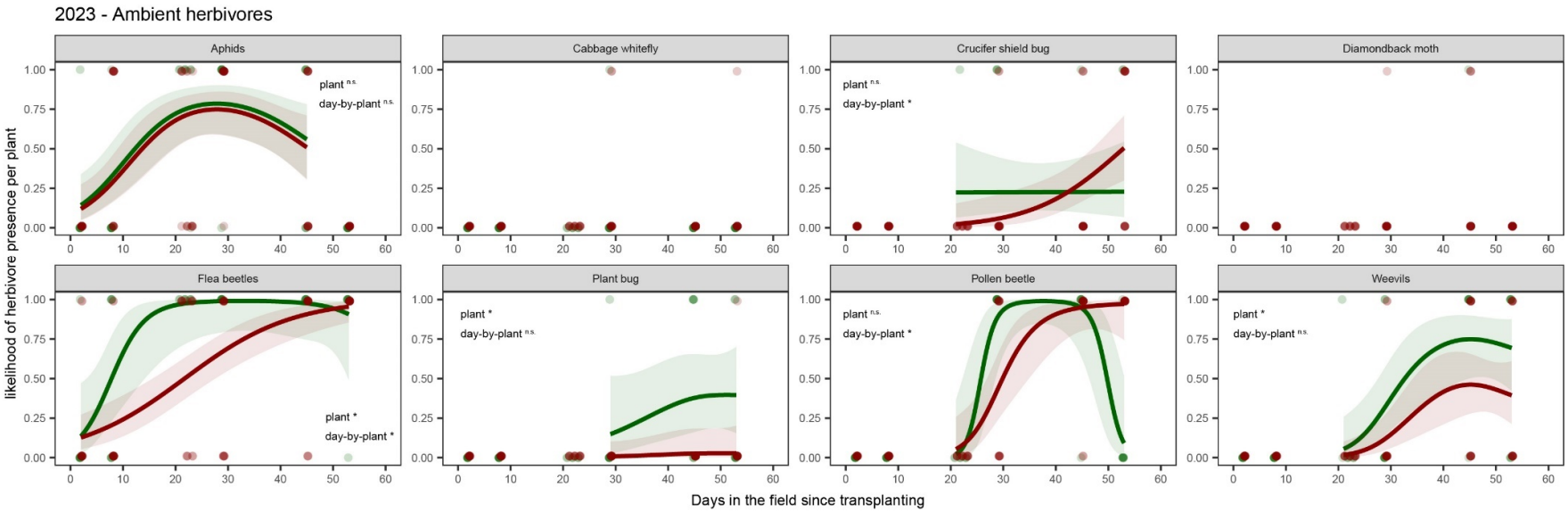

Figure S4. Likelihood of herbivore presence per plant over 9 weeks for *B. nigra* (green) and *E. cheiranthoides* (red) in the 2023 field experiment. The X-axis shows the days since plants were placed in the field, and the y-axis shows the likelihood of herbivore presence per plant. Points are observations on individual plants and lines are model fits from generalized additive models (GAMs). Shaded areas are the 95% confidence intervals. For the cabbage whitefly and the diamondback moth, observed numbers were too low to fit a reliable GAM. In each panel, 'plant' refers to statistical support of a main effect of plant species, and 'day-by-plant' for support of a species-specific smoother. See Table S3 for details.

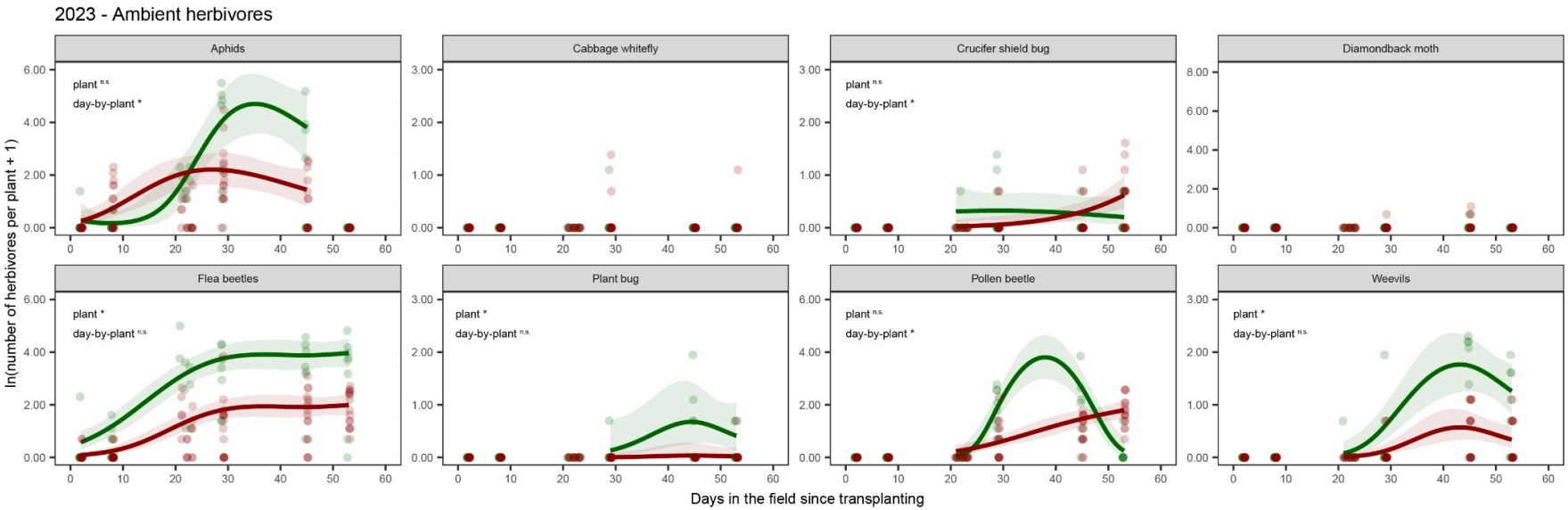

Figure S5. Number of herbivores per plant over 9 weeks for *B. nigra* (green) and *E. cheiranthoides* (red) in the 2023 field experiment. The X-axis shows the days since plants were placed in the field, and the y-axis shows the natural logarithm of the number of herbivores per plant. Points are observations on individual plants and lines are model fits from generalized additive models (GAMs). Shaded areas are the 95% confidence intervals. For the cabbage whitefly and the diamondback moth, observed numbers were too low to fit a reliable GAM. In each panel, 'plant' refers to statistical support of a main effect of plant species, and 'day-by-plant' for support of a species-specific smoother. See Table S3 for details.

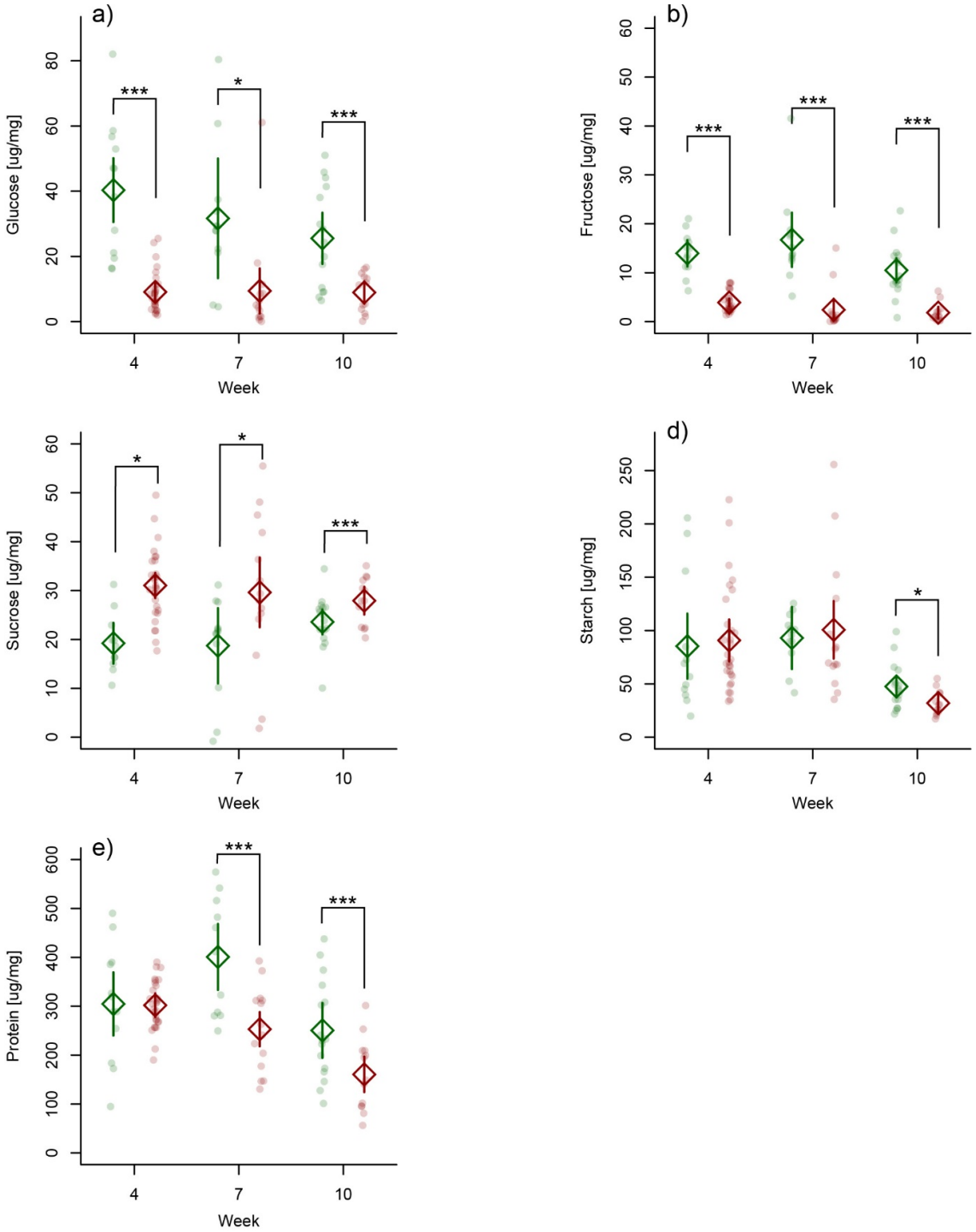

Figure S6. Concentrations of soluble sugars (a-c), starch (d), and total protein (d) in leaf tissue of *B. nigra* (green) and *E. cheiranthoides* (red) harvested in weeks 4, 7, and 10 of the field experiment. Points are values for individual plants, while diamond symbols and lines are means and 95% confidence intervals from a linear model. Brackets highlight significant linear contrasts at the $p < 0.05$ level.

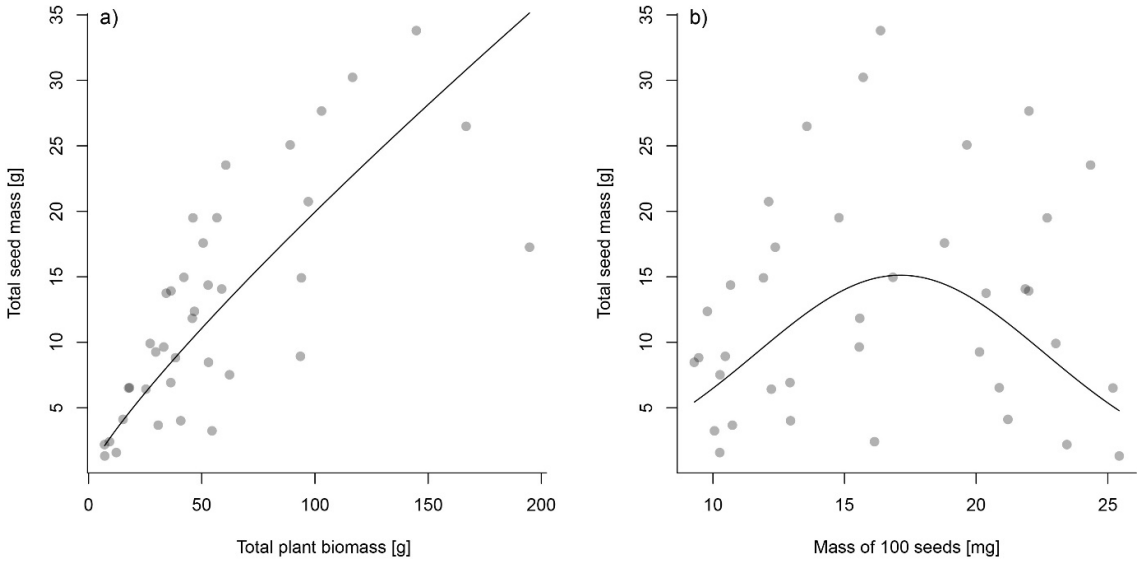

Figure S7. a) Relationship between total seed mass and aboveground plant biomass of a subset of *E. cheiranthoides* plants (2022 field experiment, block 7) that was removed from the field intact and maintained through seed ripening in a greenhouse. Points are values for individual plants, each belonging to a different accession. The solid line is a linear fit between log-transformed seed mass and log-transformed total biomass ($F_{1,36} = 77.48$, $p<0.001$, $R^2 = 0.67$.) b) Relationship between total seed mass and mass of individual seeds, showing a non-linear relationship of total seed mass and the mass of 100 seeds, indicating a potential seed size/ seed number trade-off. The solid line is a quadratic fit between log-transformed seed mass and the combined mass of 100 seeds (quadratic term: $F_{1,35} = 7.15$, $p=0.011$, $R^2 = 0.12$).