

# Flowers prepare themselves: leaf and root herbivores induce specific changes in floral phytochemistry with consequences for plant interactions with florivores

Quint Rusman<sup>1,3</sup> , Sanne Hooiveld-Knoppers<sup>1</sup>, Mirjam Dijksterhuis<sup>1</sup>, Janneke Bloem<sup>1</sup>, Michael Reichelt<sup>2</sup> , Marcel Dicke<sup>1</sup>  and Erik H. Poelman<sup>1</sup> 

<sup>1</sup>Laboratory of Entomology, Wageningen University & Research, Droeveendaalsesteeg 1, Wageningen 6708PB, the Netherlands; <sup>2</sup>Department of Biochemistry, Max Planck Institute for Chemical Ecology, Hans-Knöll-Strasse 8, 07745 Jena, Germany; <sup>3</sup>Present address: Department of Systematic and Evolutionary Botany, University of Zürich, Zollikerstrasse 107, 8008 Zürich, Switzerland

## Summary

Author for correspondence:  
Quint Rusman  
Email: [quint.rusman@systbot.uzh.ch](mailto:quint.rusman@systbot.uzh.ch)

Received: 17 May 2021  
Accepted: 6 December 2021

*New Phytologist* (2022) **233**: 2548–2560  
doi: 10.1111/nph.17931

**Key words:** *Brassica nigra*, flower-feeding organisms, flowers, glucosinolates, herbivore-induced plant responses, phenotypic plasticity, phytohormones, plant defence.

- The phenotypic plasticity of flowering plants in response to herbivore damage to vegetative tissues can affect plant interactions with flower-feeding organisms. Such induced systemic responses are probably regulated by defence-related phytohormones that signal flowers to alter secondary chemistry that affects resistance to florivores. Current knowledge on the effects of damage to vegetative tissues on plant interactions with florivores and the underlying mechanisms is limited.
- We compared the preference and performance of two florivores on flowering *Brassica nigra* plants damaged by one of three herbivores feeding from roots or leaves. To investigate the underlying mechanisms, we quantified expression patterns of marker genes for defence-related phytohormonal pathways, and concentrations of phytohormones and glucosinolates in buds and flowers.
- Florivores displayed contrasting preferences for plants damaged by herbivores feeding on roots and leaves. Chewing florivores performed better on plants damaged by folivores, but worse on plants damaged by the root herbivore. Chewing root and foliar herbivory led to specific induced changes in the phytohormone profile of buds and flowers. This resulted in increased glucosinolate concentrations for leaf-damaged plants, and decreased glucosinolate concentrations for root-damaged plants.
- The outcome of herbivore–herbivore interactions spanning from vegetative tissues to floral tissues is unique for the inducing root/leaf herbivore and receiving florivore combination.

## Introduction

A major constraint for plants in maximizing their fitness is their exposure to antagonists during plant ontogeny. Plants need to optimize prevailing interactions with community members that first colonize vegetative tissues such as roots and leaves, as well as organisms that later interact with reproductive organs such as flowers and fruits (Poelman & Kessler, 2016; Barton & Boege, 2017; Rusman *et al.*, 2019a). One of the strategies that plants evolved to deal with temporal variation in herbivore attack is phenotypic plasticity (Agrawal, 2001). Plants readily induce local and systemic phenotypic changes in response to attack by herbivores (Poelman & Dicke, 2014; Stam *et al.*, 2014; Biere & Goverse, 2016), which aim to repel or kill the attackers and/or reduce the fitness consequences of damage (Karban & Baldwin, 1997). From work in particular on plants in the vegetative stage it has become clear that such responses are often systemic, have community-wide effects and influence plant evolution (Ohgushi, 2005, 2016; Poelman &

Kessler, 2016; Mertens *et al.*, 2021a). The evolutionary consequences of herbivore-induced plant responses may be most profound if a response in the vegetative parts of the plant also affects reproductive organs and their interactions with antagonists and mutualists (McArt *et al.*, 2013; Rusman *et al.*, 2019a, 2020), because changes in reproductive organs and their associated interactors can directly alter the fertilization and consumption of ovules, which are tightly connected to fitness. During flowering, floral tissues are damaged by insects ranging from generalists that nibble bits of various floral tissues to highly specialized feeders that only feed on pollen or seeds (McCall & Irwin, 2006). Following the natural dynamics of herbivore attack in the field, plants need to defend themselves from vegetative-tissue-feeding herbivores while preparing for impending attack on the flowers (Boaventura *et al.*, 2021; Mertens *et al.*, 2021a).

To defend against flower feeders, plants arm flowers with similar types of direct defences as in leaves, such as chemical deterrents, toxins and physical barriers (McCall & Irwin, 2006). In agreement

with the optimal defence theory (McKey, 1974), flowers often contain higher constitutive levels of defences compared to vegetative tissues (Zangerl & Rutledge, 1996; Strauss *et al.*, 2004; Keith & Mitchell-Olds, 2017). Moreover, flowers can show high levels of plasticity in response to herbivores (Parachnowitsch *et al.*, 2019; Rusman *et al.*, 2019a). Damage by flower-feeding herbivores can induce phenotypic changes in floral tissues such as increases and decreases in concentrations of plant-defence-related phytohormones (Chrétien *et al.*, 2018), defence compounds (Zangerl & Rutledge, 1996; Boyer *et al.*, 2016) and changes in floral volatiles, as well as the colour and size of flower display (Lucas-Barbosa *et al.*, 2016; Rusman *et al.*, 2019b). These traits are important for host-plant selection and/or the growth of florivores (Theis & Adler, 2012; McCall *et al.*, 2013; Boaventura *et al.*, 2021). Damage by flower-feeding herbivores can thereby affect the preference and performance of con- and heterospecific florivores, and their natural enemies (McCall, 2006; Chrétien *et al.*, 2018). Interestingly, damage to vegetative tissues such as roots and leaves can also affect plant interactions with flower feeders (Ikemoto *et al.*, 2017; McCall *et al.*, 2018; Rusman *et al.*, 2018, 2020) and florivore network dynamics (Stam *et al.*, 2018). To understand the ecological and evolutionary implications of root/folivore–florivore interactions for plants, it is important to investigate whether the outcome of such interactions varies for different herbivores feeding on roots, leaves or flowers, as well as the underlying mechanisms such as vegetative-to-reproductive tissue induction.

Herbivore-induced plant responses that underlie plant-mediated herbivore–herbivore interactions involve phytohormonal signalling, gene expression and production of defensive compounds. Research on flower development suggests that the gene regulatory network for phytohormones involved in plant defence, and especially jasmonic acid (JA), is conserved in vegetative and floral tissues (Rusman *et al.*, 2019a). Flower and leaf herbivory can induce the expression of JA biosynthesis genes and increase JA concentrations in the flowers (McArt *et al.*, 2013; Zhou *et al.*, 2017; Chrétien *et al.*, 2018; Peng *et al.*, 2018). Since the backbone of defence signalling seems conserved in leaves and flowers, we can fall back on what is known regarding the specificity of defence induction in leaves: chewing and root herbivores mainly induce the JA pathway while sap-feeding herbivores induce the salicylic acid (SA) pathway and/or suppress JA induction (Ali & Agrawal, 2012; Erb *et al.*, 2012; Thaler *et al.*, 2012). The JA and SA signalling pathways show crosstalk, which generally results in reciprocal antagonism (Thaler *et al.*, 2012), but not always (Li *et al.*, 2016; Mertens *et al.*, 2021b). Such crosstalk has consequences for plant-mediated herbivore–herbivore interactions: JA induction by chewing herbivores may make plants more resistant to other chewing herbivores but more susceptible to sap-feeding herbivores (Rodríguez-Saona *et al.*, 2010; Soler *et al.*, 2012; Eisenring *et al.*, 2018). Sap-feeding herbivores such as aphids may make plants more resistant to other aphids (Züst & Agrawal, 2016), but more susceptible to chewing herbivores (Rodríguez-Saona *et al.*, 2005; Zhang *et al.*, 2009). Due to the systemic nature of herbivore-induced plant responses, JA–SA crosstalk is expected to occur between leaves and flowers as well, although this has not been tested. Flowers differ from leaves and even vary among flower

organs in the accumulation and regulation of JA and SA signalling pathway components (Hause *et al.*, 2000; Rusman *et al.*, 2019a), concentrations of defence chemistry (Onodera *et al.*, 2014; Palmer-Young *et al.*, 2019; Stegemann *et al.*, 2019) and primary metabolites (Abdalsamee & Müller, 2015), which might reduce JA–SA crosstalk. A mechanistic understanding of the consequences of herbivory on vegetative tissues for plant interactions with florivores, and more specifically whether flower tissues reflect local or systemic species-specific induction patterns, will help us understand the evolution of integrated defence responses across plant tissues (Agrawal & Fishbein, 2006).

Here, we investigated whether systemic plant defence responses are specific for herbivore attack on roots vs leaves of flowering plants and the consequences for plant interactions with florivores. We specifically studied how herbivore attack by three different herbivores feeding on roots and leaves of black mustard (*Brassica nigra*) plants affected the preference and performance of two florivores, the phytohormonal profiles and expression of related marker genes in buds and flowers, and concentrations of the main class of defence compounds of brassicaceous plants, glucosinolates. We expected root and foliar herbivores to induce systemic plant defences in flowering plants, thereby changing phytohormone and defence compound concentrations in buds and flowers. We expected chewing root and leaf herbivores to induce the JA pathway and increase resistance to chewing florivores, while leaf-feeding aphids were expected to induce the SA pathway and increase resistance to florivorous aphids.

## Materials and Methods

### Plant and insects

Seeds of black mustard (*Brassica nigra* L., accession no. CGN06619) were obtained from the Centre for Genetic Resources (CGN, Wageningen, the Netherlands), and propagated for > 10 yr by natural pollination and exposed to natural conditions in the experimental fields around Wageningen University. Seeds were germinated in trays. One-week-old plants were transplanted and cultivated in pots (Ø 17 cm, 2 l) filled with potting soil (Lentse potgrond) and sand in a 1 : 1 volume ratio under glasshouse conditions (23 ± 2°C, 50–70% relative humidity (RH), 16 h : 8 h, light : dark). Once plants started flowering (5–6 wk old) they were used in the experiments.

We used five herbivore species routinely reared in the Laboratory of Entomology (Wageningen University) in our experiments: the cabbage root fly (*Delia radicum* L.), turnip sawfly (*Athalia rosae* L.), turnip aphid (*Lipaphis erysimi* Kaltentbach), large cabbage white (*Pieris brassicae* L.) and cabbage aphid (*Brevicoryne brassicae* L.). Larvae of the cabbage root fly were used as root-feeding herbivores, larvae of the turnip sawfly and adult turnip aphids were used as folivores, while caterpillars of the large cabbage white and adult cabbage aphids were used as florivores. Although all four aboveground herbivore species can feed on leaves and flowers of *B. nigra*, caterpillars of the large cabbage white and wingless adult cabbage aphids actively migrate to flowers when placed on leaves of flowering plants, while larvae of the

turnip sawfly and wingless adult turnip aphids stay to feed on the leaves (Smallegange *et al.*, 2007; Bandeili & Müller, 2010) (Q. Rusman, pers. obs.). In the field, larvae of the turnip sawfly and turnip aphids are often found early in the season on leaves, while cabbage aphids are found later in the season in large numbers on the flowers (Mertens *et al.*, 2021b). Cabbage root flies were reared on rutabaga (*Brassica napus*) in a climate cabinet ( $22 \pm 1^\circ\text{C}$ , 50–70% RH, 16 h : 8 h, light : dark). The turnip sawfly and turnip aphid were reared on *Raphanus sativus* plants, and the large cabbage white and cabbage aphid on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) under glasshouse conditions ( $22 \pm 1^\circ\text{C}$ , 50–70% RH, 16 h : 8 h, light : dark).

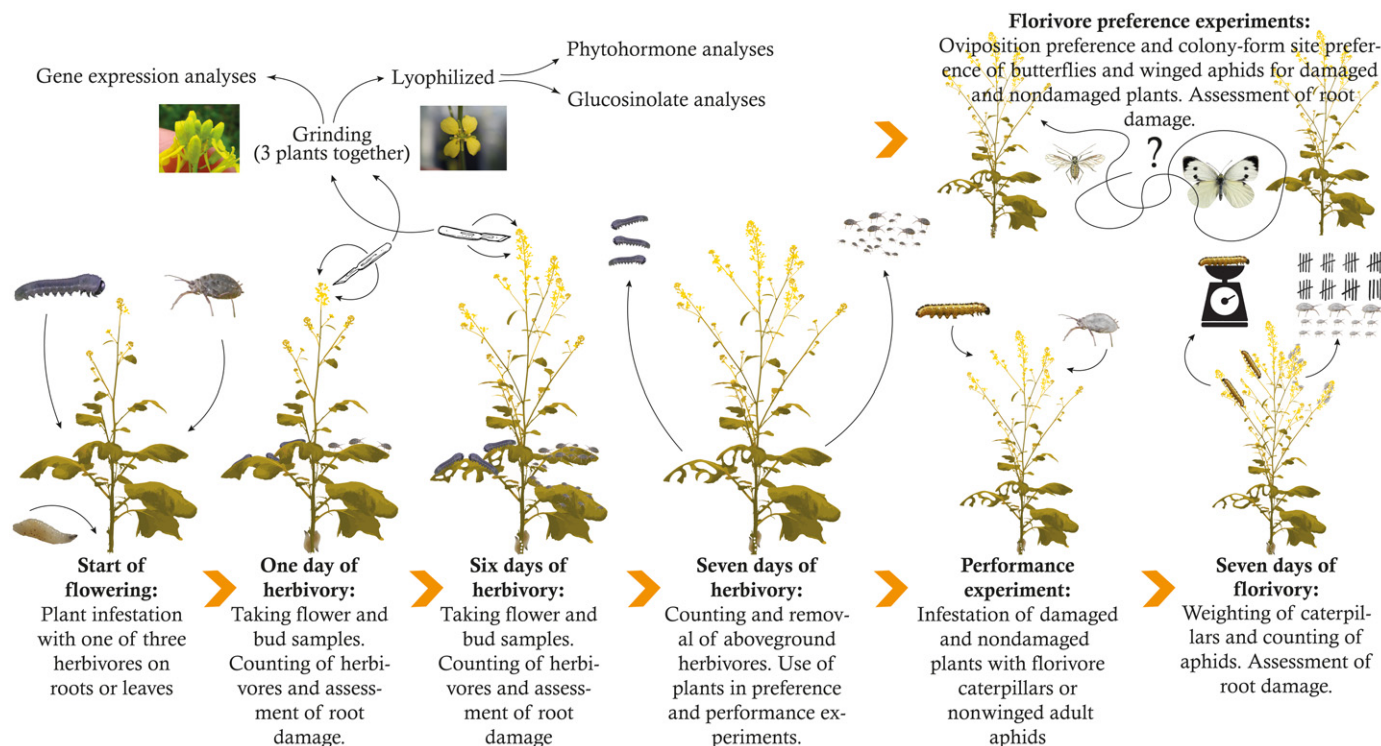
### Plant treatments

We infested early flowering *B. nigra* plants (1–3 d after opening of the first flower) with one of three herbivores: the root fly *D. radicum*, the sawfly *A. rosae* and the aphid *L. erysimi*. First-instar root fly larvae ( $n=10$ ) were placed on the soil at the base of the stem. First-instar sawfly larvae ( $n=10$ ) and wingless adult aphids ( $n=20$ ) were equally distributed over two true leaves (5–10 per leaf respectively), and the third and fourth leaves as counted from below were used for infestation. To prevent insects from moving between vegetative and flowering parts of the plant, we attached cotton wool with a small piece of wire around the main stem between the vegetative and flowering part of all plants (Rusman *et al.*, 2019c). Herbivore infestation densities were based on

average numbers of individuals from different feeding guilds per plant over multiple years of season-long field observations (Chrétien *et al.*, 2018; Rusman *et al.*, 2018, 2020; Mertens *et al.*, 2021b), as well as pilot experiments and experience in the glasshouse (Rusman *et al.*, 2019b,c). Depending on the experiment, plants were used after 1, 6 or 7 d of herbivore feeding (Fig. 1).

### Plant interactions with florivores

To investigate whether plant interactions with florivores were affected by root and foliar herbivory, we measured the preference and performance of two florivores, *P. brassicae* and *B. brassicae*. Damaged and undamaged plants were used after 7 d of root or foliar herbivore feeding, and all aboveground herbivores were removed and counted (Supporting Information Fig. S1). This was done to avoid direct effects of the leaf herbivores on the florivores, especially during the host plant preference testing. In addition, aphid populations tend to reach extremely large numbers after prolonged time in the glasshouse without top-down control. This would have become a problem for the leaf aphid treatment when kept on the plant during florivory. It is not uncommon to observe such relatively short-term periods of herbivore presence on plants in the field (Q. Rusman, pers. obs.), probably due to plant defence induction, predation and herbivore dispersal. Unfortunately, it was not possible to remove the belowground herbivores without severely damaging/affecting the plants.



**Fig. 1** Schematic representation of the timeline of the experiments. Different sets of *Brassica nigra* plants were used for the sampling after 1 and 6 d of herbivory, and for the different preference and performance experiments. Gene expression, phytohormone and glucosinolate analyses were performed on samples from the same plants. Plants were infested with one of three herbivores: the root herbivore *Delia radicum*, and foliar herbivores *Athalia rosae* or *Lipaphis erysimi*. As florivores we used *Pieris brassicae* and *Brevicoryne brassicae*. Photo credits: Jitte Groothuis, Dani Lucas-Barbosa, Daan Mertens, Quint Rusman.

Therefore, root damage was checked after plants were used in the preference and performance experiments. Root damage was checked by digging up and washing the roots, and visually estimating the percentage root tissue (tap and lateral roots) surface damage (Fig. S1).

The preference of florivores was tested by offering butterflies or winged adult aphids two plants: an undamaged plant and a plant damaged by one of the three root/foliar herbivores. For butterflies, the experiment was carried out in a flight chamber set-up (gauze tent of 293 × 200 × 230 cm) in a glasshouse compartment (25 ± 1°C, 50–70% RH, 16 h : 8 h, light : dark). A single mated female butterfly was released at a time, at 100 cm and perpendicular from the plants which were 80 cm apart (Lucas-Barbosa *et al.*, 2016). We recorded landing preference (plant of first contact), oviposition preference (plant on which eggs were laid), time between landing and oviposition on the plant of final choice, and the total time spent per plant. When butterflies chose a plant and started to oviposit, they were quickly disrupted and caught. In case an egg was laid, it was immediately removed. When a butterfly did not make a choice within 5 min, it was recorded under 'no response', and the observation was terminated. Butterflies were 3–6 d old, provided with 10% honey solution and used only once in the experiment. We ensured we only used mated females by placing 1–3 d old virgin male and female butterflies together, collecting the mating pairs and using females 1–3 d after mating (Lucas-Barbosa *et al.*, 2016; Rusman *et al.*, 2019b). For each plant pair, eight to 10 responsive butterflies were observed. For each herbivore treatment, eight or nine plant pairs were tested. For aphids, the plant pair (*c.* 50 cm apart) was placed in a mesh tent (95 × 95 × 190 cm) in a glasshouse compartment (23 ± 1°C, 50–70% RH, 16 h : 8 h, light : dark). Twenty winged aphids were placed in a Petri dish (Ø 9 cm) on top of a wooden pedestal (height 38 cm); this pedestal stood *c.* 50 cm from the plants (Rusman *et al.*, 2019c). Twenty-four hours after release, aphids on both plants were counted and their feeding site (vegetative tissues; young leaves, old leaves, stems and inflorescence tissues; developing pods, buds, flowers, bracts and floral stems) was recorded. Aphids recorded elsewhere in the tent (*i.e.* not on the plants) were recorded as being nonresponsive. For each herbivore treatment, nine to 12 plant pairs were tested.

To measure performance, we infested undamaged and herbivore-damaged plants with 10 late first-instar *P. brassicae* caterpillars or 20 wingless *B. brassicae* adults. Insects were carefully selected to be of approximately similar age and size. Insects were placed on the buds and flowers, on which they directly start feeding, of the final inflorescence of the two or four top flowering branches per plant, with five individuals per inflorescence. After 7 d of feeding, the number of aphids per plant was estimated as described in Rusman *et al.* (2019c), and caterpillars were weighed. Experiments were carried out in a glasshouse compartment (23 ± 1°C, 50–70% RH, 16 h : 8 h, light : dark).

### Floral defence signalling in flowers

To investigate whether floral defence signalling was induced by herbivore attack to roots and leaves, we measured gene expression

and phytohormone concentrations in floral tissues of herbivore-damaged and undamaged plants. One day and 6 d after herbivore feeding, all buds or up to 10 open flowers from the main inflorescence were harvested starting from the most recently opened flower down the inflorescence. Samples were wrapped in aluminium foil, immediately frozen in liquid nitrogen and stored at –80°C. Aboveground herbivores were counted and root damage was checked by digging up and washing the roots, and counting damage spots (for 1 d of feeding) or visually estimating the percentage root tissue (tap and lateral roots) surface damage (for 6 d of feeding).

As a measure of plant-defence responses at the molecular level, we quantified expression levels of two marker genes in *B. nigra*: *LIPOXYGENASE 2 (LOX2)* and *PATHOGENESIS-RELATED PROTEIN 1 (PRI)*. *LOX2* is a gene in the JA biosynthesis pathway and used as a marker gene for herbivore-induced activation of the JA signalling pathway in leaves (Bell & Mullet, 1993; Zheng *et al.*, 2007). *PRI* is a defence-related gene downstream of SA biosynthesis and used as a marker gene for herbivore-induced activation of the SA signalling pathway in leaves (Pieterse *et al.*, 2012). From floral tissues we extracted RNA using the Isolate II plant RNA kit (Bioline, Memphis, TN, USA). Tissues of three plants were ground together to make one biological replicate. After extraction, RNA concentrations were quantified using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA; all samples with an OD 260/280 nm of 1.9–2.2 ratio). Samples were diluted and adjusted to an RNA concentration of 85 ng µl<sup>-1</sup>. From the RNA samples, cDNA was synthesized using the SensiFAST cDNA synthesis kit (Bioline). Expression levels of each sample were quantified by reverse transcriptase quantitative PCR (RT-qPCR) (CFX96 Touch™ Real-Time PCR Detection System; Bio-Rad) using the SensiFAST SYBR no-ROX kit (Bioline). We added 5 µl of cDNA to the reaction with a total volume of 25 µl. Two technical replicates were used for each sample. Samples were omitted from further analyses if the expression difference between technical replicates was > 0.5. Plate setups included three interrun calibrators and a negative control (no template). In addition to the two marker genes, we analysed the expression levels of three reference genes: *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)*, *ACTIN-2 (ACT2)* and *ELONGATION FACTOR 1-α (EF1-α)*. Gene-expression data were imported to QBASE+ 3.1 (Biogazelle, Zwijnaarde, Belgium), to calculate the calibrated normalized relative quantity (CNRQ) for each gene per sample. The CNRQ value represents the relative gene expression calculated by correcting the measured gene expression of the target genes with the expression values of the reference genes and interrun calibrators.

As a measure of plant defence responses at the hormonal level, we quantified phytohormone profiles in floral tissues of *B. nigra*. Tissues of three plants were ground together to make one biological replicate and lyophilized (Snijders type 4020 lyophilizer; Tilburg, the Netherlands). Ten to 20 mg of lyophilized plant material was extracted with 1 ml methanol solution containing 40 ng D4-SA, 40 ng D6-ABA (both Santa Cruz Biotechnology, Santa Cruz, CA, USA), 40 ng D6-JA and 8 ng D6-JA-Ile (both

HPC Standards GmbH, Borsdorf, Germany) for phytohormone quantification, and 50 nmol of 4-hydroxybenzyl glucosinolate for quantification of glucosinolates (see 'Glucosinolates in flowers'). Phytohormone analysis was performed by LC-MS/MS as in Vadassery *et al.* (2012), and a full description can be found in Methods S1.

### Glucosinolates in flowers

To investigate if floral defences were induced by root and foliar herbivory, we quantified glucosinolate concentrations in flowers and buds of *B. nigra*. The same samples used to quantify phytohormone profiles were used to quantify glucosinolate concentrations by high-performance liquid chromatography (HPLC)-UV. A 600  $\mu$ l aliquot of the methanol raw extract for phytohormone analysis (see the 'Floral defence signalling in flowers' section) was loaded onto DEAE Sephadex A 25 columns and treated with arylsulfatase for desulfation (Sigma-Aldrich) (Brown *et al.*, 2003). The eluted desulfoglucosinolates were separated using HPLC (Agilent 1100 HPLC system; Agilent Technologies, Santa Clara, CA, USA) on a reversed phase C-18 column (Nucleodur Sphinx RP, 250  $\times$  4.6 mm, 5  $\mu$ m; Machrey-Nagel, Düren, Germany) with a water (A)–acetonitrile (B) gradient (0–1 min, 1.5% B; 1–6 min, 1.5–5% B; 6–8 min, 5–7% B; 8–18 min, 7–21% B; 18–23 min, 21–29% B; 23–23.1 min, 29–100% B; 23.1–24 min 100% B and 24.1–28 min 1.5% B; flow 1.0 ml min<sup>-1</sup>). Detection was performed with a photodiode array detector and peaks were integrated at 229 nm. Desulfated glucosinolates were identified by comparison of retention time and ultraviolet (UV) spectra to those of purified standards previously extracted from *Arabidopsis thaliana* (Brown *et al.*, 2003) or by analysis of the desulfoglucosinolate extracts on an LC-ESI-Ion-Trap-mass spectrometer (Esquire6000; Bruker Daltonics, Billerica, MA, USA). We used the following molar response factors for quantification of individual glucosinolates relative to the internal standard, 4-hydroxybenzyl glucosinolate: aliphatic glucosinolates 2.0, indole glucosinolates 0.5 (Burow *et al.*, 2006), 2-phenylethyl glucosinolates 2.0.

### Statistical analyses

To test if butterfly preference (first choice of landing and oviposition, time spent per plant, and oviposition time) differed between undamaged plants and plants damaged by root/leaf herbivores, we used the proportion of the response variable between damaged and undamaged plants (Rusman *et al.*, 2019b,c). We used generalized linear mixed models (GLMMs) with a binomial distribution and a log link function. The response variable was fitted to the intercept, and plant pair was used as a random factor. For caterpillar weight, we used a GLMM with a Gamma distribution with log link function to correct for model assumption violations. Inducing herbivore species was included in the models as a fixed factor. The fixed factor 'inducing herbivore species' tested for different insect/plant responses to the inducing herbivores and unfested control plants. Plant ID and trial were initially used as random factors.

To test whether aphid preference (number of aphids per plant) differed between undamaged and plants damaged by root/leaf herbivores, we used the proportion of the response variable between damaged and undamaged plants (Rusman *et al.*, 2019b, c). We used generalized linear models (GLMs) with a binomial distribution and a log link function, and the response variable was fitted to the intercept. To test whether aphid preference for plants, and plant tissues and organs (vegetative tissues; young leaves, old leaves, stems and inflorescence tissues; developing pods, buds, flowers, bracts and floral stems) differed between undamaged plants and plants damaged by root/leaf herbivores, we ran two models. The first model included inducing herbivore species and plant tissue (leaves/flowers) as fixed factors. The second model included inducing herbivore species and plant organ as fixed factors. A single nested model with inducing herbivore species and plant organ nested in plant tissue could not be run due to oversaturation of the model. For both models, we used GLMs with a Poisson distribution and a log link function or negative binomial distribution with a log link function to correct for overdispersion. Both models included the total number of aphids (responsive and unresponsive) as offset (Rusman *et al.*, 2019c). For aphid performance, we used a GL(M)M with a negative binomial distribution with a log link function to correct for overdispersion. We used inducing herbivore species as a fixed factor and initially included trial as a random factor.

To test whether phytohormone profiles differed between buds and flowers of undamaged plants and plants damaged by root/leaf herbivores, we used permutational multivariate analyses of variance (PERMANOVAs). Data were log transformed to achieve a symmetrically distributed data set, and range scaled to account for differences in compound variation (Hervé *et al.*, 2018). In a first overall analysis, inducing herbivore species, plant tissue (buds/flowers) and time point were included in the model as fixed factors. Because we were mostly interested in the effects of inducing herbivore species, and because the overall analysis identified large differences between tissues and varying effects of herbivory over time (significant interaction effects of time point with both inducing herbivore species and plant tissue; Table S5), we performed separate analyses per plant tissue and time point with inducing herbivore species as a fixed factor. We used Bray–Curtis dissimilarity index and 1000 permutations in all PERMANOVAs. For *post hoc* analysis, we performed pairwise comparisons for all pairs of levels of a factor (PERMANOVA-PAIR). Visualization of phytohormone profiles was done with projection to latent structures discriminant analysis (PLS-DA).

For gene expression, and phytohormone and glucosinolate concentrations we used (G)LMs with a Gaussian distribution and identity link function or a Gamma distribution with log link function if the data did not follow a normal distribution. Inducing herbivore species, plant tissue and time point were included in the model as fixed factors, as well as all possible interactive effects. The fixed factor 'plant tissue' tested for tissue-specific differences/responses of buds and flowers. For all (G)L(M)Ms, likelihood ratio tests were used to derive *P*-values. For all *post hoc* analyses, we performed Tukey's *post hoc* tests. All analyses were carried out in R (v.3.5.1  $\times$  64, 2018, The R Foundation for

Statistical Computing Platform). Functions and packages used are described in Methods S1.

## Results

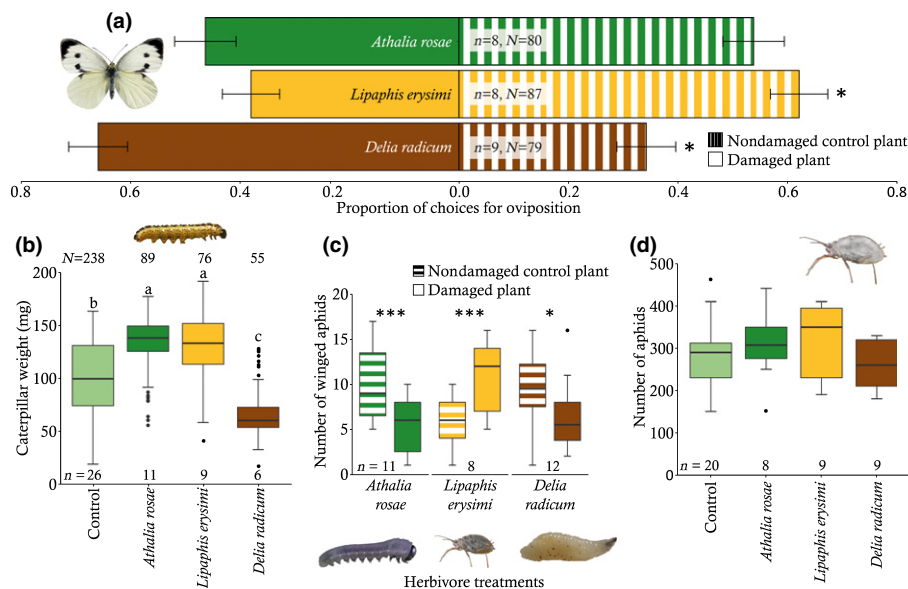
### Preference and performance of florivores on induced plants

Preference for damaged vs undamaged plants and performance on both plant types differed for both florivore species, *P. brassicae* and *B. brassicae*. These effects were species-specific for both the three herbivore species used to damage plants and the two responding florivorous species. *Pieris brassicae* butterflies preferred to land and lay eggs on *D. radicum* root-damaged plants over undamaged plants (Fig. 2a; Table S1). While butterflies preferred to lay eggs on undamaged plants when offered a choice with *L. erysimi* aphid-damaged plants, they landed similarly often on both plant types (Table S1). Butterflies did not prefer to land or lay eggs when offered undamaged plants vs plants damaged by leaf-chewing *A. rosae* larvae (Table S1). None of the three inducing herbivores affected the time spent per plant or the time needed to lay an egg by *P. brassicae* (Fig. S2; Table S1). The performance of *P. brassicae* caterpillars on flowers was affected by previous herbivory on plants (Fig. 2b, GLMM:  $df=3$ ,  $\chi^2=20.25$ ,  $P<0.001$ ). Caterpillars gained more weight when feeding on floral tissues of plants damaged by *A. rosae* or *L. erysimi* compared with undamaged plants, while they gained less weight when feeding on plants damaged by *D. radicum* (Fig. 2b; Table S2).

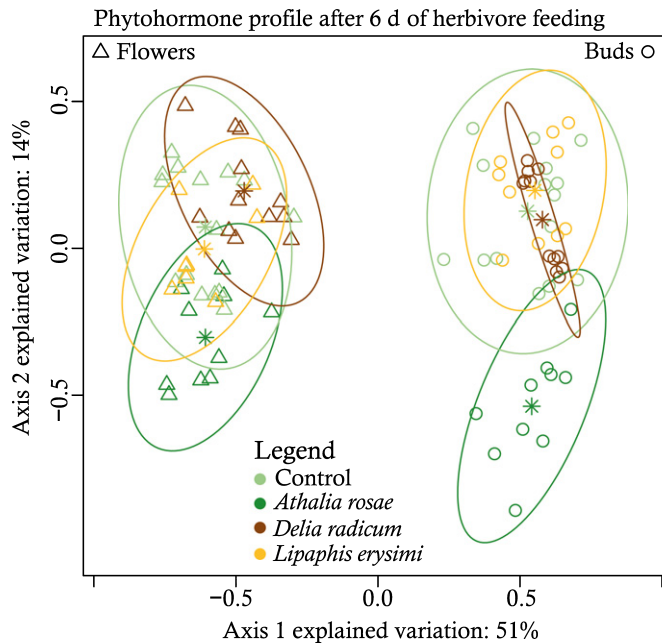
Winged *B. brassicae* aphids preferred to settle on *L. erysimi* aphid-damaged plants over undamaged plants, while aphids preferred to settle on undamaged plants when offered vs *A. rosae*-chewing- or *D. radicum* root-damaged plants (Fig. 2c; Table S1). Aphids chose specific feeding sites on plants: they preferred to settle on the inflorescences compared with vegetative tissues (Table S3). Within inflorescence tissues, aphids preferred to settle on the buds over other organs (Fig. S3; Table S3). Overall, herbivory did not affect the specific within-plant feeding site choice of aphids: no significant interactions between herbivory and plant tissue/organ were found (Fig. S3; Table S4). The performance of *B. brassicae* aphids was not affected by herbivory (Fig. 2d, GLM:  $df=3$ ,  $\chi^2=3.50$ ,  $P=0.321$ ).

### Floral defence signalling in flowers

The phytohormonal profiles of buds and flowers differed significantly (Figs 3, S4; Table S5, PERMANOVA:  $R^2=60$ ,  $df=1$ ,  $P<0.001$ ). This difference could largely be explained by a higher abundance of JA and jasmonoyl-L-isoleucine (JA-Ile) in buds compared with flowers (Fig. S5). Overall, concentrations of JA and JA-Ile were respectively 9949 and 931% higher in buds compared with flowers (Table S6). Differences between buds and flowers in activity of the JA pathway were further supported by a 65% higher relative expression of *LOX2*, and 32 and 6% higher concentrations of respectively 12-oxo-phytodienoic acid (OPDA) and hydroxy-JA-Ile (OH-JA-Ile) in buds (Fig. S5; Table S6). By



**Fig. 2** Preference and performance of florivores on plants damaged by different herbivores (*Athalia rosae*, *Lipaphis erysimi* and *Delia radicum*) and undamaged *Brassica nigra* plants. (a) Oviposition preference as the proportion of *Pieris brassicae* butterflies (mean  $\pm$  SE) that choose between plants damaged by different herbivores and undamaged plants. (b) Performance of *P. brassicae* caterpillars on floral tissues of plants damaged by different herbivores and undamaged plants. (c) Feeding site preference expressed as the number of winged *Brevicoryne brassicae* aphids found 24 h after release on plants damaged by different herbivores and undamaged plants. (d) Performance of *B. brassicae* aphids on floral tissues of plants damaged by different herbivores and undamaged plants.  $n$  = number of plant (pair) replicates,  $N$  = number of butterflies/caterpillars. Boxplots show median (line), 1<sup>st</sup> and 3<sup>rd</sup> quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1<sup>st</sup> or above the 3<sup>rd</sup> quartile) are represented by circles. Asterisks indicate significant differences between damaged and undamaged plants within a plant pair: \*\*\*,  $P<0.001$ ; \*,  $0.01 \leq P \leq 0.05$ . Letters above bars indicate significant differences at  $P \leq 0.05$  based on Tukey's *post hoc* tests. Photo credits: Jitte Groothuis, Daan Mertens, Quint Rusman.



**Fig. 3** Project to latent structures discriminant analysis (PLS-DA) of phytohormone profiles of floral tissues of damaged and undamaged *Brassica nigra* plants. Profiles include marker gene expressions and concentrations of phytohormones, precursors and breakdown products. Profiles were measured in buds (circles) and flowers (triangles) after 6 d of herbivory by larvae of the sawfly *Athalia rosae*, *Lipaphis erysimi* aphids or larvae of the cabbage root fly *Delia radicum* as well as for uninfested control plants. Profiles include the upstream jasmonic acid (JA) marker gene *LIPOXYGENASE 2* (*LOX2*), JA-precursor 12-oxo-phytyldienoic acid (OPDA), JA and jasmonoyl-L-isoleucine (JA-Ile), and JA catabolites hydroxy-JA (OH-JA), hydroxy-JA-Ile (OH-JA-Ile) and carboxy-JA-Ile (COOH-JA-Ile), salicylic acid (SA), the downstream SA marker gene *PATHOGENESIS-RELATED PROTEIN 1* (*PR1*) and abscisic acid (ABA). Gene expression was expressed as calibrated normalized relative quantity (CNQR); relative gene expression was calculated by correcting the measured gene expression of the target genes with the expression values of the reference genes and interrun calibrators. Phytohormone concentrations are expressed as  $\text{ng g}^{-1}$  of dry plant biomass. Asterisks denote centroids. Highlighted areas denote 95% confidence intervals. The number of replicates per herbivore treatment varied between eight and 16.

contrast, concentrations of SA, abscisic acid (ABA) and hydroxy-JA (OH-JA) were respectively 132, 14 and 62% higher in flowers than in buds, and relative expression of the marker gene *PR1* for SA signalling was 123% higher in flowers than in buds (Figs S5, S6; Table S6).

Herbivory affected the phytohormonal profile of buds and flowers (Fig. 3; Table S5). The effects depended on herbivore species and the duration of herbivory (Table S5). One day of herbivore feeding induced significant changes in the phytohormonal profiles of flowers but not of buds (PERMANOVA: buds  $R^2 = 4.7$ ,  $df = 3$ ,  $P = 0.823$ , flowers  $R^2 = 11.3$ ,  $df = 3$ ,  $P = 0.034$ ). The profiles of plants damaged by root-feeding *D. radicum* differed from plants damaged by *L. erysimi* aphids, but not from undamaged plants or plants damaged by leaf-chewing *A. rosae* (PERMANOVA-PAIR:  $P = 0.030$ ,  $P = 0.349$ ,  $P = 0.183$  respectively). Six days of herbivore feeding induced significant changes in the phytohormonal profiles of both buds and flowers (PERMANOVA: buds  $R^2 = 14.0$ ,  $df = 3$ ,  $P = 0.003$ , flowers  $R^2 = 18.6$ ,  $df = 3$ ,  $P < 0.001$ ). The profiles of

plants damaged by *A. rosae* differed from undamaged plants (PERMANOVA-PAIR: buds  $P = 0.030$ , flowers  $P = 0.003$ ), plants damaged by *L. erysimi* (PERMANOVA-PAIR: buds  $P = 0.030$ , but not flowers  $P = 0.484$ ) and *D. radicum* (PERMANOVA-PAIR: buds  $P = 0.006$ , flowers  $P = 0.003$ ). This difference could largely be explained by a higher abundance of OH-JA and OH-JA-Ile for plants infested with *A. rosae* compared with the other treatments (Tables 1, S2; Fig. S5). The profiles of plants damaged by *D. radicum* differed from undamaged plants (PERMANOVA-PAIR: flowers  $P = 0.030$ ) and *L. erysimi*-damaged plants (PERMANOVA-PAIR: buds  $P = 0.030$ , flowers  $P = 0.039$ ).

The three herbivores induced specific changes in the concentrations of several markers for activity of the JA pathway (Fig. S5; Tables 1, S5). Expression of the JA marker gene *LOX2* and concentrations of the JA precursor OPDA were higher in buds and flowers of plants damaged by root-feeding *D. radicum* compared with most other treatments (Fig. S5a,b; Tables 1, S2). Concentrations of OPDA were lower for plants damaged for both 1 and 6 d by leaf-chewing *A. rosae* (Fig. S5b; Tables 1, S2). Although concentrations of JA and JA-Ile itself were not affected, herbivory did affect concentrations of several breakdown products of JA and JA-Ile (Fig. S5c,d; Tables 1, S5). Concentrations of OH-JA and OH-JA-Ile were higher in buds and flowers of plants damaged for 6 d by *A. rosae* compared with most treatments (Fig. S5e,f; Tables 1, S2). Concentrations of OH-JA-Ile in buds 6 d after feeding were also higher for plants damaged by *D. radicum* compared with undamaged and plants damaged by *L. erysimi* aphids (Fig. 5f; Tables 1, S2).

Although concentrations of SA were not affected by herbivory, the expression of a downstream marker gene for the SA pathway, *PR1*, was (Fig. S6; Tables 1, S5). Flowers of plants damaged for 1 d by leaf-chewing *A. rosae* and *L. erysimi* aphids upregulated *PR1* expression, and for *A. rosae*-damaged plants this was also the case in buds (Fig. S6a; Tables 1, S2). Buds and flowers of plants damaged for 6 d by root-feeding *D. radicum* downregulated *PR1* expression (Fig. S6b; Tables 1, S2). Herbivory did not significantly affect concentrations of ABA (Fig. S6c).

### Glucosinolates in flowers

We found four glucosinolate compounds in the buds and flowers of black mustard: sinigrin, 4-hydroxy-glucobrassicin, glucobrassicin and gluconasturtiin. Sinigrin constituted 98.6% of the total concentration of glucosinolates, 4-hydroxy-glucobrassicin 0.8%, glucobrassicin 0.1% and gluconasturtiin 0.6%. Overall, total glucosinolate concentrations were 6.3% higher in flowers compared with buds (Fig. S7; Table S6). Flowers contained 6.3% more sinigrin, 53.8% more glucobrassicin and 11.5% more gluconasturtiin compared with buds (Fig. S8; Table S6).

Herbivory induced species-specific changes in the concentration of total glucosinolates and each of the glucosinolate compounds in buds and flowers (Figs S7, S8; Tables 2, S7). Buds and flowers of plants damaged by leaf-chewing *A. rosae* contained higher concentrations of total glucosinolates and sinigrin compared with the other treatments, while plants damaged by root-feeding *D. radicum* contained lower concentrations of

**Table 1** Genes and compounds involved in jasmonic acid (JA) and salicylic acid (SA) pathway signalling in floral tissues of damaged and undamaged *Brassica nigra* plants.

| Floral defence signalling | LOX2 |    | OPDA    |    |      |    | OHJA    |    | OHJA-Ile |    |         |    | PR1  |    |         |    |   |
|---------------------------|------|----|---------|----|------|----|---------|----|----------|----|---------|----|------|----|---------|----|---|
|                           | Buds |    | Flowers |    | Buds |    | Flowers |    | Buds     |    | Flowers |    | Buds |    | Flowers |    |   |
|                           | d1   | d6 | d1      | d6 | d1   | d6 | d1      | d6 | d1       | d6 | d1      | d6 | d1   | d6 | d1      | d6 |   |
| <i>Athalia rosae</i>      | .    | .  | .       | .  | -    | -  | -       | -  | .        | +  | .       | +  | .    | +  | +       | .  | + |
| <i>Lipaphis erysimi</i>   | .    | .  | .       | .  | .    | .  | .       | .  | .        | .  | .       | .  | .    | .  | .       | .  | + |
| <i>Delia radicum</i>      | +    | +  | +       | +  | +    | +  | +       | +  | .        | .  | .       | .  | .    | .  | .       | -  | - |

Gene expression and concentrations were measured in buds and flowers after 1 and 6 d of herbivory by larvae of the sawfly *Athalia rosae*, *Lipaphis erysimi* aphids, or larvae of the cabbage root fly *Delia radicum* as well as for uninfested control plants. Increase (+), decrease (–) or no effect (.) when compared to trait expression in uninfested plants. Only genes and compounds that differed between damaged and undamaged plants are included. Compound structure is jasmonic acid. LOX2: LIPOXYGENASE 2, OPDA: 12-oxo-phytodienoic acid, JA-Ile: jasmonoyl-L-isoleucine, OH-JA: hydroxy-JA, OH-JA-Ile: hydroxy-JA-Ile, and COOH-JA-Ile: carboxy-JA-Ile, PR1: PATHOGENESIS-RELATED PROTEIN 1.

**Table 2** Glucosinolates in floral tissues of damaged and undamaged *Brassica nigra* plants.

Floral defence chemistry

| Floral defence chemistry | Total glucosinolates |    |         |    | Sinigrin |    |         |    | 4-Hydroxy-glucobrassicin |    |         |    | Gluconasturtiin |    |         |    | Glucobrassicin |    |         |    |
|--------------------------|----------------------|----|---------|----|----------|----|---------|----|--------------------------|----|---------|----|-----------------|----|---------|----|----------------|----|---------|----|
|                          | Buds                 |    | Flowers |    | Buds     |    | Flowers |    | Buds                     |    | Flowers |    | Buds            |    | Flowers |    | Buds           |    | Flowers |    |
|                          | d1                   | d6 | d1      | d6 | d1       | d6 | d1      | d6 | d1                       | d6 | d1      | d6 | d1              | d6 | d1      | d6 | d1             | d6 | d1      | d6 |
| <i>Athalia rosae</i>     | +                    | +  | +       | +  | +        | +  | +       | +  | .                        | .  | .       | .  | .               | +  | .       | +  | .              | .  | .       | .  |
| <i>Lipaphis erysimi</i>  | .                    | .  | .       | .  | .        | .  | .       | .  | .                        | .  | .       | .  | .               | .  | .       | .  | .              | .  | .       | .  |
| <i>Delia radicum</i>     | -                    | -  | -       | -  | -        | -  | -       | -  | -                        | -  | -       | -  | -               | -  | .       | .  | .              | .  | .       | -  |

Glucosinolates were measured in buds and flowers after 1 and 6 d of herbivory by larvae of the sawfly *Athalia rosae*, *Lipaphis erysimi* aphids, or larvae of the cabbage root fly *Delia radicum* as well as for uninfested control plants. Increase (+), decrease (–), or no effect (.) when compared to trait expression in uninfested plants. Compound structure is sinigrin.

glucosinolates than undamaged plants (Figs S7, S8; Tables 2, S2). Buds and flowers of plants damaged by *D. radicum* also contained lower concentrations of 4-hydroxy-glucobrassicin and glucobrassicin (only for flowers) compared with the other treatments (Fig S8; Tables 2, S2). For gluconasturtiin, buds and flowers of plants damaged for 1 d by *D. radicum* or *L. erysimi* aphids contained lower concentrations compared with undamaged plants and for plants damaged by *D. radicum* also compared with *A. rosae*-damaged plants (Fig. S8; Tables 2, S2). By contrast, buds and flowers of plants damaged for 6 d by *A. rosae* contained higher concentrations of gluconasturtiin compared with the other treatments (Fig. S8; Tables 2, S2).

## Discussion

Our results show that flowering plants reshape their floral phytochemistry differentially in response to three herbivore species

attacking roots or shoots. These changes corresponded to changes in plant interactions with two species of florivores. Effects on the preference and performance of florivores were not in agreement with JA–SA crosstalk: chewing florivores preferred root-herbivore-damaged plants over undamaged plants but preferred undamaged plants over plants damaged by foliar-feeding aphids. By contrast, aphid florivores preferred plants damaged by foliar-feeding aphids over undamaged plants but preferred undamaged plants over chewing-herbivore-damaged plants. At odds with preference, caterpillar florivores performed better on plants damaged by foliar herbivores and worse on plants damaged by root herbivores, while aphid florivore performance was not affected. Induction patterns in buds and flowers partially agreed with JA–SA crosstalk: floral induction of the JA pathway and related glucosinolate defences by the leaf chewer *A. rosae*, while these were not activated by the leaf phloem-feeding aphid *L. erysimi*. The root chewer *D. radicum* induced the JA pathway differently



compared to *A. rosae*, leading to lower glucosinolate defences. As a result, the outcome of herbivore–herbivore interactions spanning from vegetative tissues to floral tissues is characterized by specificity for the inducing root/leaf herbivore and receiving florivore.

Predicting the outcome of plant-mediated herbivore–herbivore interactions and thereby understanding the underlying mechanisms has proven notoriously difficult (Biere & Goverse, 2016). We show here that JA–SA crosstalk does not predict the outcome (combined preference and performance) of herbivore–herbivore interactions spanning from vegetative to reproductive tissues. This is in line with recent findings on vegetative cross-resistance for *B. nigra* (Mertens *et al.*, 2021b) and *B. oleracea* (Li *et al.*, 2016, 2018). The choice behaviour exhibited by our florivores in most cases did not result in increased larval growth, as predicted by the ‘mother knows best’ hypothesis (Thompson, 1988; Gripenberg *et al.*, 2010; McCall *et al.*, 2018). While correlated preference–performance is expected with JA–SA crosstalk, the herbivore-induced plant phenotype did not affect florivore preference and performance in the same direction. Factors other than JA–SA crosstalk to explain the outcome of herbivore–herbivore interactions are: the identity of the second herbivore, that is how well that herbivore species can deal with induced plant defences (Agrawal, 2000; Mertens *et al.*, 2021b); modulation of the induced plant responses (Erb *et al.*, 2012), for example by herbivore feeding site (de Rijk *et al.*, 2016; Rusman *et al.*, 2019c); other plant physiological processes such as nutrient allocation dynamics and investment tradeoffs (Johnson *et al.*, 2016; Züst & Agrawal, 2017); and prevalence of herbivores in the field – that is, are the herbivore species common or uncommon and are plants adapted to employ induced defences to cooccurring herbivores (Mertens *et al.*, 2021a,b)?

The importance of the identity of the second herbivore offers a plausible explanation for the observed preference behaviour in our study. The two florivore species use different flower traits to find a suitable flowering host plant: *P. brassicae* butterflies use flower size and flower volatiles, such as phenylacetaldehyde (Knauer & Schiestl, 2017), while *B. brassicae* aphids use colour (yellow and UV) and unknown volatiles (Döring, 2014). Leaf and root herbivory result in distinct floral volatile profiles for *B. nigra*, including the emission of phenylacetaldehyde, as well as changes in yellow and UV reflection of flowers (Rusman *et al.*, 2019b). Herbivory thereby changes the strength of the cues that florivores use in host plant selection, while additional information comes from, for example, predation risk (Dannon *et al.*, 2010; Schiestl *et al.*, 2014; Silveira *et al.*, 2018). Importantly, such herbivore-induced changes in flower traits do not match patterns predicted by JA–SA crosstalk, but depend on herbivore identity (Rusman *et al.*, 2019b) and feeding site (Lucas-Barbosa *et al.*, 2016; Rusman *et al.*, 2019c). The specific trait-use by florivores to find a suitable host plant and how these traits are affected by root and leaf herbivory provide a more accurate explanation than JA–SA crosstalk for the observed preference outcome of herbivore–herbivore interactions.

Interestingly, the feeding site of the inducing herbivore appears to be a good predictor of the effects on chewing florivore

performance, and this corresponds partly with patterns of glucosinolate concentrations after induction. Root herbivory reduced the performance of chewing florivores and resulted in lower glucosinolate concentrations in buds and flowers. Leaf herbivory increased the performance of chewing florivores, and leaf chewing herbivory resulted in higher glucosinolate concentrations in floral tissues. The data suggest that chewing florivores somehow benefit from higher glucosinolate concentrations, while aphid florivores do not benefit but were also not negatively affected. Both florivores are specialists on brassicaceous plants and adapted to the glucosinolate defence system, but employ different detoxification mechanisms (Winde & Wittstock, 2011; Jeschke *et al.*, 2016). The glucosinolate profile of buds and flowers of *B. nigra* consists for almost 99% of the aliphatic glucosinolate sinigrin. Sinigrin and total aliphatic glucosinolate concentrations have been shown to vary in their effects on specialist aphids and caterpillars (Cole, 1997; Agrawal & Kurashige, 2003; Mewis *et al.*, 2005; Gols *et al.*, 2008; Müller *et al.*, 2010; Kos *et al.*, 2012a,b; Santolamazza-Carbone *et al.*, 2016). It is currently not known whether detoxification of secondary defence compounds can be beneficial to specialist herbivores by extracting valuable nutrients, but this has been suggested (Stauber *et al.*, 2012). Our results suggest that such benefits might differ depending on the detoxification mechanism employed, and this is an exciting question for future research.

In contrast to the outcome of herbivore–herbivore interactions, traits of the inducing herbivore and specifically the feeding guild predict plant trait induction relatively well (Textor & Gershenzon, 2009; Bidart-Bouzat & Kliebenstein, 2011; Ali & Agrawal, 2012). We show here that vegetative-to-reproductive tissue induction partially agrees with JA–SA crosstalk, that is differential plant induction by herbivores of various feeding guilds. Changes in the concentrations of JA precursors and breakdown products in response to herbivory by the leaf chewer *A. rosae* suggest that the JA pathway was activated (Textor & Gershenzon, 2009). Increased concentrations of the JA pathway markers *LOX2* and *OPDA* due to herbivory by the root herbivore *D. radicum* suggest slow or delayed activation of the JA pathway. Aphids seem to induce no strong systemic responses in floral defence chemistry. Our results align with recent studies on induction of phytohormones by chewers and aphids in local floral (Chrétien *et al.*, 2018) and systemic root tissues (Karssemeijer *et al.*, 2020). In addition to differences in feeding mode/site, leaf and root herbivory also differed in treatment length. Lasting limited herbivore damage may induce different plant responses than short intense nibbles (Underwood, 2010, 2012). Coinfestations by root and flower herbivores may have differentially affected the performance of florivores compared to sequential solo infestations (Johnson *et al.*, 2012). However, the inherent slow growth of belowground herbivores probably led to a slower accumulation of damage and a slower or delayed plant response, as suggested by our results. In the field, coinfection between below- and aboveground herbivores are likely to be common, while leaf and flower feeder cooccurrence might be more stochastic, especially because many leaf feeders move to flowers when these become available (Smallegange *et al.*, 2007; Agerbirk *et al.*, 2010; Bandelli

& Müller, 2010). Plant responses should reflect herbivore feeding characteristics and cooccurrence patterns in the field (Mertens *et al.*, 2021a), and therefore different plant responses rather than differences in treatment length *per se* probably explain the differences in florivore performance on plants damaged by chewing leaf and root herbivores.

A fundamental question regarding the systemic induction of inflorescence tissues by herbivory on vegetative tissues is why the responses are systemic at all. The prevalence of systemic plant responses suggests such responses to be adaptive, for two possible reasons. First, vegetative-to-reproductive tissue induction may help plants deal with current attackers. Many herbivores start feeding on leaves but later move to flowers (Lucas-Barbosa *et al.*, 2013; Abdalsamee & Müller, 2015; Tsuji *et al.*, 2018). Second, vegetative-to-reproductive tissue induction may be adaptive by affecting other community members (Poelman & Kessler, 2016; Rusman *et al.*, 2019a). When leaf damage provides a reliable cue for future floral damage, inducing floral defences upon leaf herbivory may reduce negative fitness impacts of florivory (Karban *et al.*, 1999; Adler *et al.*, 2006; Mertens *et al.*, 2021a). For example, foliar herbivory can increase seed production by inducing resistance to seed predators via increased levels of JA and phenolic defence compounds (McArt *et al.*, 2013). In our case, root/leaf herbivory by chewing herbivores repelled the aphid florivore *B. brassicae* but attracted the chewing florivore *P. brassicae*. In the field, *B. brassicae* is a much more common florivore than *P. brassicae* (Mertens *et al.*, 2021b). Hence, inducing floral defences that repel the most common florivore upon exposure to a cue (leaf/root herbivory) that herbivores are present/abundant seems an adaptive response.

Our study shows that root and leaf herbivory change plant resistance to florivores potentially via vegetative-to-reproductive tissue induction. Understanding the specificity of plant-mediated interactions and the resulting (ecological) costs and benefits for plant fitness is instrumental to understanding plant defence evolution (Poelman & Kessler, 2016; Erb, 2018). Plant fitness is determined by the cumulative fitness effects of plant interactions with herbivores during the vegetative and flowering stages. Our study shows that herbivory during these stages is not independent but connected through systemic plant responses. When damage types covary, for example because florivores prefer root- and/or leaf-damaged plants, we can expect selection for covariation of (induced) leaf and flower defence traits, which ultimately results in the evolution of integrated defence syndromes across roots, leaves and flowers (Agrawal & Fishbein, 2006; Mertens *et al.*, 2021a). When vegetative-to-reproductive tissue induction leads to ecological costs such as susceptibility to florivores, we may expect a reduced or complete disconnection of vegetative-to-reproductive tissue induction. However, we show that vegetative-to-reproductive tissue induction involves highly conserved signalling pathways, and the extent to which plants can disconnect such responses is open to question (Rusman *et al.*, 2019a; Kessler & Chautá, 2020).

## Acknowledgements





We thank Peter Karssemeijer for much appreciated help in the laboratory, and Jonathan Gershenzon for insightful discussion.

QR was supported by the Earth and Life Science Council of the Netherlands Organisation for Scientific Research (NWO-ALW; grant no. 831.14.004). EHP was funded by the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme (grant agreement no. 677139).

## Author contributions

QR and EHP planned and designed the study. QR, SH-K, M Dijksterhuis and JB collected insect data, plant tissue samples and plant gene expression data. MR performed phytohormone and glucosinolate analyses. QR analysed the data. QR, M Dicke and EHP interpreted the data and wrote the manuscript.

## ORCID

Marcel Dicke  <https://orcid.org/0000-0001-8565-8896>  
 Erik H. Poelman  <https://orcid.org/0000-0003-3285-613X>  
 Michael Reichelt  <https://orcid.org/0000-0002-6691-6500>  
 Quint Rusman  <https://orcid.org/0000-0003-0285-7967>

## Data availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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## Supporting Information

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

**Fig. S1** Treatment intensities for *Brassica nigra* plants damaged by leaf and root herbivores measured on different timepoints.

**Fig. S2** Total time spent and oviposition time by *Pieris brassicae* butterflies on *Brassica nigra* plants damaged by different herbivores and undamaged plants.

**Fig. S3** Number of winged aphids (mean  $\pm$  SE) on various plant organs of damaged and undamaged *Brassica nigra* plants.

**Fig. S4** Project to latent structures discriminant (PLS-DA) analysis of phytohormone profiles of floral tissues damaged by different herbivores for 1 d and undamaged *Brassica nigra* plants.

**Fig. S5** Concentrations of various genes and compounds involved in the jasmonic acid signalling pathway in floral tissues of damaged and undamaged *Brassica nigra* plants.

**Fig. S6** Concentrations of SA and ABA, and relative expression of *PRI* in floral tissues of damaged and undamaged *Brassica nigra* plants.

**Fig. S7** Total glucosinolate concentrations in floral tissues of damaged and undamaged *Brassica nigra* plants.

**Fig. S8** Concentrations of sinigrin, 4-hydroxy-glucobrassicin (4OHI3M), glucobrassicin (I3M) and gluconasturtiin (2PE) in floral tissues of damaged and undamaged *Brassica nigra* plants.

**Methods S1** Full description of phytohormone analysis and description of functions and packages used in the statistical analyses.

**Table S1** Output of *post hoc* analyses for models of preferences of adult *Pieris brassicae* butterflies and winged *Brevicoryne brassicae* aphids for damaged and undamaged *Brassica nigra* plant pairs.

**Table S2** Output of *post hoc* analyses for models of *Pieris brassicae* caterpillar performance and phytochemistry of plant tissues (buds

and flowers) of *Brassica nigra* plants comparing damaged and undamaged plants.

**Table S3** Output of *post hoc* analyses for models of preferences of winged *Brevicoryne brassicae* aphids for plant tissues (vegetative or inflorescence tissues) and organs of damaged and undamaged *Brassica nigra* plant pairs.

**Table S4** Output of (generalized) linear models showing the effects of different fixed (herbivore treatment, plant tissue, plant organ) factors on plant preferences of winged *Brevicoryne brassicae* aphids for damaged and undamaged *Brassica nigra* plants.

**Table S5** Output of statistical models showing the effects of different fixed (herbivore treatment, plant tissue, time point) factors on the relative expression and concentrations of various genes and compounds involved in phytohormone signalling in floral tissues of damaged and undamaged *Brassica nigra* plants.

**Table S6** Output of *post hoc* analyses for models of phytochemistry of buds and flowers of damaged and undamaged *Brassica nigra* plants.

**Table S7** Output of (generalized) linear models showing the effects of different fixed (herbivore treatment, plant tissue, time point) factors on the concentrations of glucosinolates in floral tissues of damaged and undamaged *Brassica nigra* plants.

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