A mechanism for sequence specificity in plant-mediated interactions between herbivores

Wei Huang¹, Christelle A. M. Robert¹, Maxime R. Hervé¹,², Lingfei Hu¹, Zoe Bont¹, Matthias Erb¹*

¹ Institute of Plant Sciences, University of Bern, Altenbergrain 21, Bern 3013, Switzerland
² INRA, UMR1349 IGEPP, F-35653 Le Rheu, France
* Author for correspondence: matthias.erb@ips.unibe.ch, 41 31 631 8668 (Tel)

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Summary

1. Herbivore communities are shaped by indirect plant-mediated interactions whose outcomes are strongly dependent on the sequence of herbivore arrival. However, the mechanisms underlying sequence specificity are poorly understood.

2. We examined the mechanisms which govern sequence specific effects between two specialist maize herbivores, the leaf feeder *Spodoptera frugiperda* and the root feeder *Diabrotica virgifera virgifera*. In the field, *S. frugiperda* reduces *D. v. virgifera* abundance, but only when arriving on the plant first.

3. In behavioral experiments, *D. v. virgifera* larvae continued feeding on plants that they had infested prior to leaf infestation, but refused to initiate feeding on plants that were infested by *S. frugiperda* prior to their arrival. Changes in root-emitted volatiles were sufficient to elicit this sequence-specific behaviour. Root volatile and headspace mixing experiments showed that early arriving *D. v. virgifera* larvae suppressed *S. frugiperda* induced volatile repellents, which led to the maintenance of host attractiveness to *D. v. virgifera*.

4. Our study provides a physiological and behavioral mechanism for sequence specificity in plant-mediated interactions and suggests that physiological canalization of behaviorally active metabolites can drive sequence specificity and result in strongly diverging herbivore distribution patterns.

Keywords

above-below ground interactions, *Diabrotica virgifera virgifera*, induced resistance, physiological canalization, plant-herbivore interactions, *Spodoptera frugiperda*, volatile organic compounds, *Zea mays*
**Introduction**

Interspecific competition influences the structure, function and stability of natural and agricultural ecosystems (Loreau & de Mazancourt, 2013). For herbivorous insects, interspecific competition can occur through direct interference or through plant-mediated, indirect effects (Denno *et al.*, 1995). A growing number of studies show that plant-mediated, indirect effects are the most common form of interspecific competition between herbivores (Ohgushi, 2005; Kaplan & Denno, 2007; Xiao *et al.*, 2012; Huang *et al.*, 2013) and that they act as driving forces of herbivore community composition in nature (Kaplan & Denno, 2007; Poelman & Dicke, 2014; Stam *et al.*, 2014).

The outcome of plant-mediated interactions between herbivores is determined by a number of factors, including the identity of the attacking herbivore, the identity of the plant and the identity of the responding herbivore (Johnson & Agrawal, 2005; Wurst & van der Putten, 2007; Xiao *et al.*, 2012; Huang *et al.*, 2014). Recently, the sequence of arrival was identified as an important factor as well: Depending on which species arrives first, the effect of one herbivore on the other can change drastically. Soler *et al.* (2012) for instance observed that *Pieris brassicae* caterpillars grew bigger when feeding on *Brassica oleracea* plants that were infested by *Brevicoryne brassicae* aphids before the arrival *P. brassicae*, but not if both herbivores attacked the plant simultaneously. A recent meta-analysis on interactions between leaf and root feeding herbivores identified the sequence of arrival as a strong predictor for the directionality of effects for this type of plant-mediated interactions (Johnson *et al.*, 2012).

To date, several physiological hypotheses have been proposed that may explain sequence specificity (Erb *et al.*, 2011a; Stam *et al.*, 2014): Plant-mediated feedback-loops, overriding induction effects and physiological canalization. Plant-mediated feedback-loops occur if two herbivores sharing a host plant influence each other reciprocally (Soler *et al.*, 2012): A first arriving herbivore could then
influence the behavior and damage patterns of a second arriver by inducing physiological changes in the plant, which, by consequence, would change the plant-mediated impact of the second herbivore on the first herbivore and thereby lead to sequence specific patterns. Overriding effects occur if one herbivore elicits a plant response that is much stronger than the response of the other herbivore and thereby determines the resulting interaction (Stam et al., 2014). Physiological canalization is a phenomenon where plant responses are determined by the first arriving herbivore (Viswanathan et al., 2007). By suppressing the response that is normally elicited by a second herbivore, physiological canalization can lead to sequence-specific effects.

Behavioral mechanisms may also lead to sequence specificity (Erb et al., 2011a; Karban, 2011). Asymmetrical host acceptance for instance refers to situations where a herbivore is less likely to start feeding on a new host plant than to continue feeding on a colonized host. This is a common pattern for sedentary herbivores such as miners and gall feeders and may lead to sequence-specific effects by modulating the behavior of a herbivore differently, depending on whether it is arriving on a host plant second or whether it is already established when another herbivore arrives.

Plant physiological and herbivore behavioral mechanisms are not mutually exclusive. Asymmetrical host acceptance for instance may be favored by plant-mediated feedback-loops, overriding effects or physiological canalization: For example, a first arriving herbivore may negatively impact a second herbivore, which may decrease the capability of second herbivore to induce volatile repellents, and in turn render the plant more attractive to first herbivore. Furthermore, a first herbivore may trigger strong physiological changes in the plant which may render it attractive to itself irrespective of the potentially unattractive changes that are induced by a second arriving herbivore. Finally, a first herbivore may change the plant’s physiology in a way that makes it unresponsive to the second herbivore, which may lead to the suppression of an otherwise unattractive physiological change. So far, the contributions of the different physiological and behavioral mechanisms and their combinations to sequence specificity have not been tested experimentally. By
consequence, the drivers of sequence specificity in indirect, plant-mediated interactions are not well understood.

Here, we analyzed potential mechanisms leading to sequence-specificity by studying the effect of attack by the leaf-feeding larvae of *Spodoptera frugiperda* (J.E. Smith) on the root-feeding larvae of *Diabrotica virgifera virgifera* (LeConte) sharing maize (*Zea mays* L.) as a common host plant. Both herbivores occur on cultivated maize and its wild ancestors and cause severe damage in both agricultural and natural systems (Branson & Krysan, 1981; O'Day, 1998). They overlap spatially and temporally in the field, with their sequence of arrival varying considerably with climatic conditions and locations (Branson, 1976; O'Day, 1998). Our previous study within the same system revealed that *S. frugiperda* larvae significantly reduce the number of *D. v. virgifera* larvae feeding on maize roots in the field, but only when *S. frugiperda* larvae arrive first (Erb *et al.*, 2011a). Subsequent experiments showed that maize root systems of plants which are attacked by leaf-feeding caterpillars become highly unattractive to *D. v. virgifera* larvae, and that this effect is mediated by long and short distance host acceptance cues (Robert *et al.*, 2012a; Erb *et al.*, 2015; Lu *et al.*, 2016). In contrast, *D. v. virgifera* attack renders the plant highly attractive to conspecifics (Robert *et al.*, 2012a) and reprograms the root metabolism to become more suitable for its own development (Robert *et al.*, 2012b). Although *D. v. virgifera* decreases the performance of leaf-feeders on maize under water limiting conditions, which may lead to plant-mediated feedback loops (Erb *et al.*, 2009; Erb *et al.*, 2011b), we found no correlation between the amount of *S. frugiperda* leaf-damage and the reduction of *D. v. virgifera* performance in our previous work (Erb *et al.*, 2011a).

Based on the findings above, we hypothesized that asymmetrical host acceptance may contribute to the sequence specific interaction patterns between *D. v. virgifera* and *S. frugiperda*, and that this asymmetrical acceptance behavior may either be the result of overriding effects or physiological canalization. We therefore conducted a series of behavioral experiments to explore the impact of the sequence of arrival on host plant attractiveness and acceptability for *D. v. virgifera* larvae. We then used a
modified two-by-two-arm olfactometer to test the influence of plant volatiles on the
sequence-specific behavior of *D. v. virgifera* and to distinguish between overriding
effects and physiological canalization. Finally, we analyzed the changes in root
volatiles elicited by the different arrival sequences to test for patterns of physiological
canalization.

**Materials and Methods**

**Plants and insects**

Maize seeds (hybrid Delprim) were obtained from Delley DSP (Delley, Switzerland). They were sown individually in plastic pots (11 cm depth, 4 cm diameter) and placed in a greenhouse (26 ± 2 °C, 14 : 10 h light : dark, 55 % relative humidity). Twelve days later (henceforth called day 0), plants with three fully developed leaves were used for experiments. Eggs of *D. v. virgifera* were obtained from the USDA-ARS (Brookings, SD) and larvae were reared on freshly germinated maize plants until use. *S. frugiperda* eggs were obtained from the University of Neuchatel, (Neuchâtel, Switzerland), and the hatching larvae were reared on soy-wheat germ diet (Bio-Serv, USA) until use.

**Plant treatments**

To establish different feeding sequences and herbivore combinations, plants were randomly assigned to one of four treatments (Fig. 1a). (1) aboveground herbivory (AG): Twelve second-instar *S. frugiperda* larvae were added to the leaves of each plant at day 2; (2) belowground herbivory (BG): Six second-instar *D. v. virgifera* larvae were added into a hole (9 cm depth, 0.5 cm diameter) in the soil at the base of each plant at day 0; (3) belowground attack followed by aboveground attack (BG>AG): Six second-instar larvae of *D. v. virgifera* were added to each plant at day 0, and twelve second-instar larvae of *S. frugiperda* were added to each plant at day 2; (4) controls without herbivory (C). These treatments simulated a situation where *D. v. virgifera* larvae newly arrive on plants already infested with *S. frugiperda* (AG) or
where they can continue feeding on maize plants that are infested by conspecifics alone (BG) or by conspecifics that arrived prior to the arrival of the leaf feeder (BG>AG) (Fig. 1a). As *D. v. virgifera* larvae refuse to feed on plants that are previously attacked by *S. frugiperda* (Robert *et al.*, 2012a; Erb *et al.*, 2015; Lu *et al.*, 2016), an AG>BG treatment was not included in the experimental setups.

To prevent above- and belowground herbivores from escaping, the aboveground parts (leaves of maize plants) were caged with transparent 1.5L plastic bottles with their bottoms removed that were put upside down on the pots. Belowground parts (pots) were covered with aluminum foil. All plants were caged the same way regardless of herbivore treatment. Furthermore, small holes were made in the soil of each plant regardless of *D. v. virgifera* infestation. Four days after the beginning of the different treatments (day 4), the plastic bottles and *S. frugiperda* larvae were removed. Then, the responding *D. v. virgifera* larvae were introduced into the system as described below (Fig. 1b-e). Timing and herbivore densities were chosen to match earlier studies and to mimic natural occurrence patterns in the field (Erb *et al.*, 2011a; Robert *et al.*, 2012a).

**Influence of sequence of arrival on host plant acceptance by *D. v. virgifera***

In a first set of experiments, we tested the hypothesis that *D. v. virgifera* larvae may reject roots of plants that are previously infested with *S. frugiperda*, but may continue to feed on plants on which they were able to establish a suitable feeding environment prior to the arrival of the leaf-feeder. We conducted experiments using three different setups, as described below (Fig. 1b-d).

First, we tested the behaviour of *D. v. virgifera* using a Petri dish setup which allowed for direct root contact (Robert *et al.*, 2012c) (Fig. 1b). The root systems of plants from the different treatment groups were gently washed with tap water. Plants were then paired in the following combinations: (1) C vs AG; (2) C vs BG; (3) C vs BG>AG. Root systems of the different plant pairs were placed on a moistened filter paper in a Petri dish (13.5 cm diameter, 2 cm depth), which had a gap (0.8 cm width,
2 cm height) on the side. The stems were laid into the gap, with the leaves remaining outside of the Petri dish. Six second-instar larvae were then added onto the moistened filter paper. The larvae could move and feed freely on the plants within the Petri dish. The Petri dish was covered with aluminum foil to decrease the impact of light on the roots and insects. The position of the larvae was recorded at 0.5h, 1.5h, 3h and 5h. Larvae that remained on the filter paper and did not choose a plant were counted as no-choice. Each treatment combination was repeated 24-36 times.

Second, we specifically tested the contribution of volatile cues to the observed behavioral patterns. For this purpose, the same treatment combinations as in the first experiment were offered to *D. v. virgifera* larvae in two-arm olfactometers as described (Robert *et al.*, 2012a) (Fig. 1c). Before the beginning of the treatments, plants were transplanted individually into L-shaped glass pots (11 cm depth, 5 cm diameter) with a horizontal connector at a height of 0.5 cm and filled with moist sand. At day 4, the horizontal connector of each glass pot was attached with one Teflon connector (29 / 32 to 24 / 29 mm) which contained a fine metal screen (2300 mesh; Small Parts Inc.). Then, the two Teflon connectors were linked using a glass tube (24 / 29 mm; length 8 cm) with a vertical access port in the middle. To keep the root systems in the dark and to avoid visual cues for the larvae, the entire olfactometer was covered with aluminum foil. Twenty minutes after connecting the different odor sources, six second-instar *D. v. virgifera* larvae were released into the access port of the glass tube. The larvae could move freely in the glass tube, but could not reach the roots of the plants. After 10 min, the olfactometer was disassembled and the number of larvae in each Teflon connector was recorded. Larvae that stayed in the central glass tube after 10 min were recorded as no-choice. For each treatment combination, 18 independent replicates were carried out.

In a third experiment, we tested whether *D. v. virgifera* larvae are more likely to leave the rhizosphere environment of infested plants, even in the absence of an alternative host. For this purpose, plants were potted and infested in L-shaped glass pots as described above (Fig. 1d). Then, six second-instar larvae were released
directly at the entrance of the horizontal access port of each glass pot. The access port of the horizontal connector was not sealed so the larvae could move into the soil and start feeding or try to escape from the plant through the access port. The L-pot was placed in a Petri dish filled with tap water at a height of 0.5 cm to catch escaping *D. v. virgifera* larvae without flooding the glass pot. The number of escaped larvae in the trap was recorded over 20 min. For each treatment, 12 replicates were carried out.

**Plant-mediated feedback-loops**

To evaluate whether belowground attack by *D. v. virgifera* changes the aboveground damage pattern by *S. frugiperda* larvae under the current experimental conditions, the leaves of plants from the different infestation treatments were collected at day 4, and total leaf area and damaged leaf area were measured for each plant using Digimizer software (MedCalc Software bvba; Mariakerke, Belgium). Eighteen replicates per treatment were carried out.

**Overriding effects**

To investigate whether an overriding signal may be responsible for the observed asymmetrical host acceptance of *D. v. virgifera* in the first set of experiments, we developed a two-by-two-arm belowground olfactometer that allowed us to combine the volatile headspaces from two odor sources per arm (Fig. 1e). For this purpose, the setup described above was modified as follows: Two Teflon connectors attached to glass pots were linked using a “Y” glass tube (24 / 29 mm; length 8 cm) at an angle of 60°. Then, two “Y” glass tubes were connected to a central glass tube (24 / 29 mm; length 8 cm) with a vertical access port in the middle. This modification enabled us to attach two L-shaped glass pots to each side of the release tubes and to test the preference of *D. v. virgifera* for two combinations of two mixed odor sources. The following treatment combinations were investigated using this setup: C+C vs C+AG; C+C vs C+BG, C+C vs AG+BG. The olfactometer was disassembled and the number of larvae in each “Y” glass tube was recorded after 10 min. We hypothesized that if *D.
v. virgifera elicits an overriding signal, the AG+BG arms should be more attractive than the C+C arm. Eighteen replicates were performed for each treatment combination.

**Physiological canalization**

To evaluate whether *D. v. virgifera* attack canalizes the root volatile response in a way that suppresses responsiveness to *S. frugiperda* infestation, we collected and analyzed root volatile profiles using solid-phase micro-extraction-gas chromatography-mass spectrometry (SPME-GC-MS). Plants were treated as described above (Fig. 1a). Crown and primary roots were then washed with tap water and frozen in liquid nitrogen. Twelve plants per treatment were harvested, and the roots of two plants were pooled for analysis, resulting in six biological replicates. The crown and primary roots of each replicate were ground into a fine powder, and 50 mg of each root type were placed in a 10 ml glass vial and sealed using Teflon tape (Polytetrafluoroethylene). An SPME fiber (100 µm polydimethylsiloxane coating, Supelco, USA) was then inserted into the vial for 60 min at 50°C. The incubated fibers were then immediately analyzed by GC-MS (Agilent 7820A GC interfaced with an Agilent 5977E MSD) following previously established protocols with a few modifications (Erb et al., 2011c). Briefly, the fiber was inserted into the injector port at 250 °C and desorbed for 2 min. After fiber insertion, the column temperature was maintained at 60 °C for 1 min and then increased to 250 °C at 5 °C min\(^{-1}\) followed by a final stage of 4 min at 250 °C. The overall analysis time for each sample, including oven cooling, was 45 min. Furthermore, to eliminate the impact of background peaks, three glass vials without any plant material (blanks) were run using the same protocol as described above. The resulting GC-MS chromatograms were processed with Progenesis QI (informatics package from Waters, MA, USA) using default settings for spectral alignment and peak picking. From the resulting matrix, all features which were presented in more than one blank were removed, resulting in 232 features. Features were assigned to individual compounds by retention time and peak shape matching and identified using the NIST search 2.2 Mass Spectral Library as well as
Data analysis

To examine host acceptance of *D. v. virgifera* in a Petri dish experiment, the number of larvae found on different herbivory treatment groups was analyzed using a Wald test applied on a Generalized Linear Mixed Model (GLMM) with a Poisson distribution. We considered plant treatment as a fixed factor, time as covariate and the replicate as a random factor. Each plant combination (C vs AG, C vs BG and C vs BG>AG) was analyzed separately. Then, to compare the preference of *D. v. virgifera* between the different treatment groups, the number of larvae on infested plants (AG, BG and BG>AG) was analyzed using a likelihood ratio test applied on a Generalized Linear Model (GLM) with a Poisson distribution. The models included herbivory as fixed factor and time as a covariate. The preference of *D. v. virgifera* larvae in the olfactometer experiments and the number of escaped larvae in escape experiment were analyzed as described above. To examine whether belowground attack by *D. v. virgifera* larvae changes the aboveground damage pattern by *S. frugiperda* larvae, the relative and absolute leaf damage of *S. frugiperda* larvae was analyzed using independent sample *t*-tests (BG vs BG>AG). The absolute leaf damage was estimated by the sum of leaf damaged area for each plant and the relative leaf damage was calculated as the sum of leaf damaged area / the sum of total leaf area × 100 for each plant. To examine the overall differences in volatile profiles, the relative abundance of the detected features were subjected to redundancy analysis (RDA) using the different treatments as a unique explanatory variable. Monte Carlo tests with 999 permutations were then used to test for significant differences between treatments. For more detailed, compound specific analyses, the different features were assigned to individual compounds, and the relative abundance of the individual compounds, which corresponds to the sum of the signal intensities of the individual features, were analyzed by one-way ANOVAs followed by least square mean *post-hoc* tests for pairwise comparisons, including false discovery rate (FDR) corrections (Benjamini & Hochberg, 1995). All analyses were conducted using R 3.2.0 (R Foundation for...
Statistical Computing, Vienna, Austria) with “car”, “lme4”, “lsmeans”, “vegan” and “RVAideMemoire” packages (Fox & Weisberg, 2011; Bates et al., 2015; Hervé, 2016; Lenth, 2016; Oksanen et al., 2016).

Results

D. v. virgifera rejects S. frugiperda infested plants only when arriving second

In the Petri dish experiment, D. v. virgifera larvae strongly preferred the roots of control plants when offered uninfested vs. leaf-infested plants ($X^2 = 30.753, P < 0.001$, Fig. 2a). By contrast, the larvae showed a strong preference for roots that were previously infested with D. v. virgifera larvae over controls ($X^2 = 69.919, P < 0.001$, Fig. 2b). Roots which were infested with D. v. virgifera two days before the onset of S. frugiperda attack remained highly attractive ($X^2 = 21.734, P < 0.001$, Fig. 2c). The number of responding D. v. virgifera larvae increased with experimental time (C vs AG, $X^2 = 5.698, P = 0.017$; C vs BG, $X^2 = 20.033, P < 0.001$; C vs BG>AG, $X^2 = 35.964, P < 0.001$; Fig. 2). At the end of the experiment, 65%, 67% and 70% of D. v. virgifera larvae made a choice in C vs AG, C vs BG and C vs BG>AG, respectively. No significant interactive effects between time and treatments were found (C vs AG, $X^2 = 3.515, P = 0.061$; C vs BG, $X^2 = 0.135, P = 0.713$; C vs BG>AG, $X^2 = 1.342, P = 0.247$). Overall, more D. v. virgifera larvae fed on BG and BG>AG roots than on AG roots ($X^2 = 38.558, P < 0.001$, Fig. 2). No difference was found between the preference of D. v. virgifera for BG and BG>AG roots ($P = 0.064$, Fig. 2).

In the two-arm olfactometer experiment, similar preference patterns were observed. D. v. virgifera larvae showed a strong preference for control plants over S. frugiperda infested plants ($X^2 = 8.111, P < 0.01$, Fig. 3). By contrast, the larvae preferred plants that were previously infested with conspecifics over controls ($X^2 = 34.177, P < 0.001$, Fig. 3). Plants were infested with D. v. virgifera prior to S. frugiperda infestation remained highly attractive ($X^2 = 16.849, P < 0.001$, Fig. 3). In this experiment, all larvae made a choice within 10 min. Together, the larvae were...
more attracted to the roots that had been infested by conspecifics alone and
conspecifics that had arrived prior to the arrival of the *S. frugiperda*, while were less
attracted to the roots that had been infested by *S. frugiperda* alone (*χ^2* = 20.396, *P* <
0.001, Fig. 3). Again, BG and AG>BG treatments were not significantly different
from each other (*P* = 0.389, Fig. 3).

When offered a single host plant, the number of escaping *D. v. virgifera* larvae
differed significantly between treatments (*χ^2* = 32.112, *P* < 0.001, Fig. 4). When
offered a *S. frugiperda* infested plant, 50% of the larvae escaped from the rhizosphere
within 20 min (Fig. 4). By contrast, less than 18% of the larvae left the soil of control
plants or plants that were previously infested with conspecifics (Fig. 4). A similar
percentage of larvae chose to remain in the rhizosphere of plants that were infested
with *D. v. virgifera* prior to *S. frugiperda* attack (Fig. 4).

**Plant-mediated feedback-loops are unlikely to explain *D. v. virgifera* behavior**

There was no significant difference in relative (*t* = 0.055, *P* = 0.957) or absolute
(*t* = 1.236, *P* = 0.225) damaged leaf area between plants from that were infested with
*D. v. virgifera* or root herbivore free (Fig. S1). These results suggest that the
interaction between *D. v. virgifera* and *S. frugiperda* is highly asymmetrical and that
plant-mediated feedback-loops are unlikely to play a major role in determining
sequence specific responses of *D. v. virgifera*.

**D. v. virgifera does not produce an overriding attractive signal**

Similarly to the two-arm olfactometer experiment, *D. v. virgifera* larvae
significantly preferred to move to the side of the olfactometer containing two control
plants rather than the arm leading to a control plant and an *S. frugiperda* infested plant
(*χ^2* = 15.446, *P* < 0.001, Fig. 5). The opposite was true for a combination of a control
plant with a *D. v. virgifera* infested plant, which was attractive to the root feeder (*χ^2* =
8.111, *P* < 0.01, Fig. 5). In contrast to the attractiveness of BG>AG plants observed in
the two-arm olfactometer experiment however (Fig. 3), the mixed rhizosphere
volatiles from an *S. frugiperda* and a *D. v. virgifera* infested plant were highly unattractive, and significantly more larvae moved to the control side ($\chi^2 = 10.333, P < 0.01$, Fig. 5) than to the AG+BG side. All larvae made a choice within the first 10 min. Overall, the presence of plants that were infested by *S. frugiperda* significantly repelled *D. v. virgifera* ($\chi^2 = 15.915, P < 0.001$, Fig. 5). This experiment falsifies the hypothesis that *D. v. virgifera* triggers an overriding attractant.

**D. v. virgifera feeding suppresses S. frugiperda induced root volatiles**

In total, we detected 232 volatile features in the GC-MS chromatograms. Redundancy analysis revealed that *S. frugiperda* and *D. v. virgifera* attack induced different volatile blends compared to control plants and compared to each other (AG vs C: $P = 0.008$; BG vs C: $P = 0.008$; BG>AG vs C: $P = 0.008$, Fig. 6). The volatile profiles of plants that were induced by *D. v. virgifera* prior to *S. frugiperda* attack were indistinguishable from plants that were infested with *D. v. virgifera* alone (BG>AG vs BG: $P = 0.642$, Fig. 6), but both of them were significantly different from plants that were infested with *S. frugiperda* alone (BG vs AG: $P = 0.008$; BG>AG vs AG: $P = 0.008$, Fig. 6). Analysis of variance revealed twelve volatile compounds whose abundance differed significantly between treatments (Fig. 7). Pairwise comparisons showed that four of these volatiles were significantly induced by *D. v. virgifera* infestation alone (Fig. 7a-d) and two of them were significantly induced by *S. frugiperda* attack alone (Fig. 7k-l). We found no significant effect of later *S. frugiperda* attack on *D. v. virgifera* induced volatile emissions (Fig. 7). However, the induction of the *S. frugiperda* induced volatiles was suppressed by early *D. v. virgifera* infestation (Fig. 7l). This result demonstrates that *D. v. virgifera* canalizes the root volatile production and renders roots unresponsive to leaf-attack by *S. frugiperda*.

**Discussion**

The sequence of arrival is increasingly recognized as an important determinant
of plant-mediated indirect interactions between herbivores (Viswanathan et al., 2005; Viswanathan et al., 2007; Poelman et al., 2008; Erb et al., 2011a; Soler et al., 2012; Wang et al., 2014). However, the mechanisms leading to sequence specificity are not well understood. The goal of the present study was to identify the (mutually non-exclusive) behavioral and physiological mechanisms that may contribute to sequence specific effects. Our experiments show that leaf attack by *S. frugiperda* strongly reduces the attractiveness of roots for *D. v. virgifera* through changes in volatile cues. However, prior *D. v. virgifera* attack suppresses these changes and thereby maintains the attractiveness of the plants to *D. v. virgifera* larvae. This form of asymmetrical host acceptance behavior explains why *S. frugiperda* reduces the abundance and damage by *D. virgifera* in the field only when arriving first on the plant (Erb et al., 2011a).

Several non-exclusive physiological mechanisms may explain why *D. v. virgifera* is repelled by *S. frugiperda* attacked plants only when arriving second. It is for instance possible that early arriving *D. v. virgifera* larvae change the behavior and induction pattern of *S. frugiperda*. However, we found no evidence for the presence of resistance feedback loops in our system: *S. frugiperda* damage remained unchanged by *D. v. virgifera* attack. Earlier studies demonstrated that *D. v. virgifera* root attack increases leaf resistance via ABA signalling under drought conditions; when plants are well watered, no negative effects of *D. v. virgifera* on *Spodoptera littoralis* growth were observed any more (Erb et al., 2011b). The maize seedlings in our experiments were supplied with sufficient soil moisture, which likely prevented potential feedback loops from occurring. Another explanation for the observed behavioural patterns is that *D. v. virgifera* may induce changes that strongly increase the attractiveness of the roots and override any negative changes that are later induced by *S. frugiperda*. By mixing volatiles from different plants, we tested this hypothesis on a behavioral level. Surprisingly, we found that *D. v. virgifera* rejected the volatile mix from a combination of plants that had been infested by *D. v. virgifera* and *S. frugiperda* separately. This is in stark contrast with the strong attractiveness of plants that were
infested with *D. v. virgifera* and *S. frugiperda* sequentially and strongly suggests that *D. v. virgifera* does not produce an overriding attractive signal.

On the other hand, our GC-MS analyses provide clear evidence that *D. v. virgifera* canalizes the plant’s root volatile response. Maize roots responded strongly to *D. v. virgifera* attack and produced higher amounts of several volatiles, including several products of the terpene synthase TPS23 which are strongly induced by *D. v. virgifera* (Köllner et al., 2008; Hiltpold et al., 2011) and attract the root feeder (Robert et al., 2012a). These responses were not altered by later *S. frugiperda* attack. By contrast, *S. frugiperda* attack induced a different set of compounds in the roots, including a yet unidentified nitrophenol, and this induction was fully suppressed by prior *D. v. virgifera* attack. These results demonstrate that early arriving *D. v. virgifera* canalizes the root metabolism in a way that makes it unresponsive to *S. frugiperda* attack. Canalization of plant responses by herbivores has been proposed to occur in a number of plant-herbivore interactions (Thaler et al., 2002; Viswanathan et al., 2005; Utsumi et al., 2010). For example, Viswanathan et al. (2007) found that tortoise beetle attack after flea beetle attack of *Solanum dulcamara* did not alter the induced resistance elicited by the flea beetles. By contrast, tortoise beetle attack before flea beetle attack resulted in the disappearance of induced resistance. One possible explanation of canalization is negative cross-talk between signaling pathways that inducing one pathway may attenuate or repress other pathways (Koornneef & Pieterse, 2008; Erb et al., 2012). Furthermore, priority in occupying a plant resource may also result in physiological canalization, as resources invested into an initial induced response may be not available for investment into later induced responses (Stam et al., 2014). In combination with the behavioral experiments, these results suggest that the asymmetrical host acceptance behavior of *D. v. virgifera* is caused by physiological canalization.

In a previous study, we found that leaf attack by *S. littoralis* leads to a slight decrease in root ethylene production, and that adding ethylene back to the root system restores the attractiveness of the roots to *D. v. virgifera* (Robert et al., 2012a). Many
herbivores increase local ethylene emissions of their host plants (Winz & Baldwin, 2001; von Dahl & Baldwin, 2007; Schäfer et al., 2011), and it is therefore possible that D. v. virgifera attack resulted in the reversal or canalization of the ethylene response of the roots. Unfortunately, ethylene emissions could not be measured in the current series of experiments. However, the presented findings suggest that S. frugiperda attack also triggers the release of repellent volatiles which are suppressed by D. v. virgifera. The escape experiment in particular shows that D. v. virgifera systematically moves away from leaf-infested plants, and it seems unlikely that a reduction in ethylene levels alone can account for this result. Furthermore, the volatile mixing experiment suggests that the volatile blend of the roots of an S. frugiperda attacked plant overrides the attractive signal from a D. v. virgifera infested root system.

In our GC-MS chromatograms, we found several volatiles which increased in the roots of S. frugiperda attacked plants. Elucidating their structure and bioactivity is an exciting prospect of this work. A recent paper identified methyl antranilate as a repellent for neonate D. v. virgifera larvae (Bernklau et al., 2016). Although methyl antranilate was not among the S. frugiperda induced root volatiles, it provides an interesting starting point to identify the volatiles which render S. frugiperda attacked plants repellent to D. v. virgifera larvae. One aspect that should be kept in mind is that root volatiles were measured by grinding root material and sampling the headspace of the ground samples by SPME. The advantages of this technique are its sensitivity and robustness. Its disadvantage is that it may result in the detection of volatile compounds which are not actually released into the rhizosphere by intact roots. Future experiments should therefore include in vivo sampling techniques to confirm the release of the newly detected volatiles into the rhizosphere (Ali et al., 2010; Hiltpold et al., 2011).

Host location and acceptance by herbivores are key processes in plant-herbivore interactions. Our results show that physiological canalization can have a strong, sequence-specific impact on host acceptance by herbivores, which may result in
strongly diverging herbivore damage and distribution patterns in the field. Our previous work shows that the repellent effect of leaf infestation on root herbivores is highly conserved across herbivore species and maize genotypes (Lu et al., 2016). Whether similar effects also occur in other plant species remains to be elucidated. Understanding the mechanisms which govern sequence specificity will allow for the integration of this phenomenon into current theory on plant-mediated interactions and will facilitate future efforts to develop predictive ecophysiological models of multi-herbivore dynamics on shared host plants.

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Figure legends

Fig. 1 Overview of the experimental design and setups used in this study. (a) Experimental treatments (infestation histories). To establish different sequences of arrival, second instar *S. frugiperda* larvae were added to the leaves, and second instar *D. v. virgifera* larvae were added to the roots of maize plants in different combinations. After 4 days of herbivore infestation, plants with different infestation histories were offered to *D. v. virgifera* larvae in choice and no-choice experiments and chemical analysis. AG: aboveground *S. frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae infestation, BG>AG: belowground infestation followed by aboveground infestation, C: control without herbivory. (b) Larval preference was measured by laying out the root systems of two plant on moist filter paper in large petri dishes. (c) Volatile-mediated larval preference was measured using a two arm belowground olfactometer. (d) Larval escape patterns were measured using a single L-shaped glass pot and a water-filled petri dish to collect the escaping larvae. (e) Volatile mixing experiments were conducted using a two arm belowground olfactometer with two volatile sources attached to each arm of the central chamber. For more details on the different treatments and setups, refer to the materials and methods section.

Fig. 2 Sequence of arrival determines root attractiveness to *D. v. virgifera*. The number of *D. v. virgifera* larvae on the roots of plants with different infestation histories was measured in Petri dish experiment. (a) *D. v. virgifera* choice between C and AG plants (n = 24). (b) *D. v. virgifera* choice between C and BG plants (n = 36). (c) *D. v. virgifera* choice between C and BG>AG plants (n = 36). AG: aboveground *S. frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae infestation, BG>AG: belowground infestation followed by aboveground infestation, C: control without herbivory. Values correspond to means ± 1 s.e. Asterisks indicate a significant difference in preference within each combination and time point (n.s., non significant; * P < 0.05; ** P < 0.01; *** P < 0.001, GLMM). Differences in preference patterns between treatment combinations are depicted by dashed lines and asterisks on the
right of the graph (n.s., non significant; *** $P < 0.001$, GLM).

**Fig. 3** Volatile cues contribute to sequence-specific preference patterns of *D. v. virgifera*. The number of *D. v. virgifera* larvae attracted to root volatiles of plants with different infestation histories was measured in two-arm olfactometers experiment. **AG:** aboveground *S. frugiperda* larvae infestation, **BG:** belowground *D. v. virgifera* larvae infestation, **BG>AG:** belowground infestation followed by aboveground infestation, **C:** control without herbivory. Values are means ± 1 s.e. ($n = 18$). Asterisks indicate a significant preference within each treatment combination (** $P < 0.01$, *** $P < 0.001$; GLMM). Different letters indicate significant differences between treatment combinations ($P < 0.05$, GLM).

**Fig. 4** Stay-or-leave patterns of *D. v. virgifera* are determined by the sequence of arrival. The number of *D. v. virgifera* larvae leaving from the rhizosphere of plants with different infestation histories was measured in escaping experiment. **AG:** aboveground *S. frugiperda* larvae infestation, **BG:** belowground *D. v. virgifera* larvae infestation, **BG>AG:** belowground infestation followed by aboveground infestation, **C:** control without herbivory. Values are means ± 1 s.e. ($n = 12$). Different letters indicate significant differences between treatments ($P < 0.05$, GLM).

**Fig. 5** Acceptances of *D. v. virgifera* are determined by the additive changes in root volatiles. The number of *D. v. virgifera* larvae attracted by mixed root volatiles from plants with different infestation histories were measured in volatile-mixing experiment, with each arm containing two different volatile sources. **AG:** aboveground *S. frugiperda* larvae infestation, **BG:** belowground *D. v. virgifera* larvae infestation, **C:** control without herbivory. Values are means ± 1 s.e. ($n = 18$). Asterisks indicate a significant preference within choice combinations (**, $P < 0.01$; *** $P < 0.001$; GLMM). Different letters indicate differences in preference patterns between treatments ($P < 0.05$, GLM).

**Fig. 6** Infestation by *D. v. virgifera* canalizes the volatile response of maize roots. The
results of a redundancy analysis (RDA) of the root volatile responses to different sequences of *D. v. virgifera* and *S. frugiperda* feeding are shown. The first two axes explained 53.86% and 24.36% of the total variation. AG: aboveground *S. frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae infestation, BG>AG: belowground infestation followed by aboveground infestation, C: control without herbivory. Data points represent individual replicates (n = 6).

**Fig. 7** *D. v. virgifera* suppresses *S. frugiperda*-induced root volatiles. The relative abundance of root volatile in four treatments were measured using solid phase micro extraction (SPME) in combination with gas chromatography and mass spectrometry (GC-MS). (a) *E*-β-Caryophyllene (17.33 min, 189.1726 m/z), (b) Humulene (18.17 min, 204.1966 m/z), (c) Unknown (19.30 min, 503.6733 m/z), (d) Unidentified Carboxylic acid (10.07 min, 123.0129 m/z), (e) Unknown (19.07 min, 173.0813 m/z), (f) Caryophyllene oxide (21.27 min, 161.1235 m/z), (g) Unknown (19.15 min, 106.0578 m/z), (h) Ethanol acetate (15.99 min, 204.1814 m/z), (i) Unknown (17.07 min, 161.0902 m/z), (j) Unknown (25.05 min, 180.0533 m/z), (k) Unknown (12.71 min, 138.0904 m/z) and (l) Unidentified nitrophenol (17.67 min, 139.0342 m/z). AG: aboveground *S. frugiperda* larvae infestation, BG: belowground *D. v. virgifera* larvae infestation, BG>AG: belowground infestation followed by aboveground infestation, C: control without herbivory. Values are means ± 1 s.e. (n = 6). Different letters indicate differences in relative abundance among treatments (*P* < 0.05, LM).

**Fig. S1.** Infestation by *D. v. virgifera* does not change aboveground damage by *S. frugiperda* larvae. Relative and absolute leaf damage caused by *S. frugiperda* on plants with and without previous infestation by *D. v. virgifera* is shown. AG: aboveground *S. frugiperda* larvae infestation, BG>AG: belowground infestation followed by aboveground infestation. Values are means ± 1 s.e. (n = 18).
Overview of the experimental design and setups used in this study

Fig. 1

190x274mm (284 x 284 DPI)
Sequence of arrival determines root attractiveness to D. v. virgifera

Fig. 2

128x216mm (300 x 300 DPI)
Volatile cues contribute to sequence-specific preference patterns of D. v. virgifera

Fig. 3

146x97mm (300 x 300 DPI)
Stay-or-leave patterns of D. v. virgifera are determined by the sequence of arrival

Fig. 4

88x78mm (300 x 300 DPI)
Acceptances of *D. v. virgifera* are determined by the additive changes in root volatiles

Fig. 5

146x101mm (300 x 300 DPI)
Infestation by D. v. virgifera canalizes the volatile response of maize roots

Fig. 6

190x142mm (300 x 300 DPI)
D. v. virgifera suppresses S. frugiperda-induced root volatiles

Fig. 7

160x213mm (300 x 300 DPI)