

Response of *Brassica oleracea* to temporal variation in attack by two herbivores affects preference and performance of a third herbivore

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Abstract. 1. Plants are frequently under attack by multiple insect herbivores, which may interact indirectly through herbivore-induced changes in the plant's phenotype. The identity, order, and timing of herbivore arrivals may influence the outcome of interactions between two herbivores. How these aspects affect, in turn, subsequently arriving herbivores that feed on double herbivore-induced plants has not been widely investigated.

2. This study tested whether the order and timing of arrival of two inducing herbivores from different feeding guilds affected the preference and performance of a subsequently arriving third herbivore, caterpillars of *Mamestra brassicae* L. (Lepidoptera: Noctuidae). Aphids [*Brevicoryne brassicae* L. (Hemiptera: Aphididae)] and caterpillars [*Plutella xylostella* L. (Lepidoptera: Yponomeutidae)] were introduced onto wild *Brassica oleracea* L. (Brassicaceae) plants in different sequences and with different arrival times. The effects of these plant treatments on *M. brassicae* caterpillars were assessed in pairwise preference tests and no-choice performance tests.

3. The caterpillars of *M. brassicae* preferred to feed from undamaged plants rather than double herbivore-induced plants. Compared with undamaged plants, they preferred plant material on which aphids had arrived first followed by caterpillars, whereas they avoided plant material with the reverse order of herbivore arrival. Performance of the caterpillars increased with increasing arrival time between herbivore infestations in double herbivore-induced plants. Although *M. brassicae* grew faster on plants induced by aphids than on those induced by caterpillars alone, its performance was not affected by the order of previous herbivore arrival.

4. These results imply that the timing of colonisation by multiple herbivores determines the outcome of plant-mediated herbivore–herbivore interactions.

Key words. Arrival sequence, *Brevicoryne brassicae*, herbivore-induced plant response, *Plutella xylostella*, arrival time, wild cabbage.

Introduction

At the basis of many aquatic and terrestrial food webs, plants typically are under attack by many herbivore species (Price,

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2002; Schmitz *et al.*, 2004; Schoonhoven *et al.*, 2005). Insect herbivores represent the most diverse group of attackers, and several species frequently co-occur on individual plants (Schoonhoven *et al.*, 2005; Stam *et al.*, 2014). Insect herbivores are rarely found to be in strong competition over plant biomass, but instead competition among these herbivores is often mediated indirectly by plant quality (Denno *et al.*, 1995; Karban & Baldwin, 1997; Kaplan & Denno, 2007; Utsumi *et al.*, 2010). Herbivores alter plant quality by inducing changes in plant traits such as growth, architecture, resource allocation and

mechanical or chemical defence properties (Hunter, 1992; Karban & Baldwin, 1997; Koricheva *et al.*, 2004; Rodriguez-Saona *et al.*, 2010). These induced plant phenotypes in turn affect the preference and performance of subsequent herbivores that interact with the herbivore-induced plant, resulting in plant-mediated interactions among herbivores (Utsumi *et al.*, 2010; Ohgushi, 2016). These plant-mediated interactions are often asymmetric (Rodriguez-Saona *et al.*, 2005; Kaplan & Denno, 2007; Miller-Pierce & Preisser, 2012; Ali & Agrawal, 2014), because plant responses to herbivores are species-specific (Agrawal, 2000; de Vos *et al.*, 2005; Viswanathan *et al.*, 2005; Kessler & Halitschke, 2007; Bidart-Bouzat & Kliebenstein, 2011; Karban, 2011) and dependent on order and timing of herbivore arrival (Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Erb *et al.*, 2011; Uesugi *et al.*, 2013; Stam *et al.*, unpublished). Asymmetry in plant-mediated interactions among herbivores may be most pronounced in herbivores of different feeding guild (Rodriguez-Saona *et al.*, 2010; Soler *et al.*, 2012), because feeding guild is a major determinant of herbivore-specific induced plant responses (de Vos *et al.*, 2005; Rodriguez-Saona *et al.*, 2010; Bidart-Bouzat & Kliebenstein, 2011). For example, caterpillars feeding on aphid-induced *Brassica oleracea* plants performed better than those feeding on control plants, while aphid performance was not affected in caterpillar-induced plants (Soler *et al.*, 2012). The performance of herbivore species is often negatively affected by plant phenotypes induced by herbivores of the same feeding guild (Rodriguez-Saona *et al.*, 2005; Mathur *et al.*, 2013).

Most herbivores will encounter a plant that already expresses a herbivore-induced phenotype, often even induced by more than a single herbivore (Karbon & Baldwin, 1997; Dicke & Hilker, 2003; Stam *et al.*, 2014). Herbivore food-plant acceptance and performance in these situations thus depend on the indirect interaction network among multiple herbivores feeding on the same plant (Utsumi *et al.*, 2010). In these interaction networks, the order and timing of herbivore arrival are particularly important, as these factors largely determine the plant phenotype expressed after attack by two herbivores. Therefore, the order and timing of herbivore arrival may have unique effects on a third herbivore in the interaction network (Viswanathan *et al.*, 2007; Utsumi *et al.*, 2010; Stam *et al.*, unpublished). For example, when a leaf-feeding herbivore arrived on maize plants before a root-feeding herbivore, the leaf-feeding herbivore negatively affected the performance of the root herbivore. In contrast, when the order of arrival was reversed, the leaf herbivore did not affect the performance of the root herbivore, suggesting that the induced plant phenotypes differ according to the order of herbivore arrival (Erb *et al.*, 2011). These effects may be modulated by the arrival time between two episodes of herbivore attack, because of temporal aspects of plant physiological responses to herbivory (Kuśnierczyk *et al.*, 2008). For example, there may be a time lag between the onset of herbivory and the onset of the plant response (Kuśnierczyk *et al.*, 2008; Gomez *et al.*, 2010; Karban, 2011). When herbivores arrive simultaneously or shortly after each other, the plant may integrate its response to both attackers. When the arrival time between arrival of herbivores is greater, the plant might already have mounted its response to the first herbivore and might have to redirect its

induced response to the second herbivore (Karbon, 2011; Underwood, 2012), potentially leading to a weaker response to the second herbivore. The kinetics of the induced plant response can also differ depending on the feeding guild of the inducer (Mathur *et al.*, 2013; Mouttet *et al.*, 2013; Kroes *et al.*, 2016). For example, a below-ground herbivore on *Plantago lanceolata* had a negative effect on an above-ground feeder, while the reverse situation, with a similar arrival time, had no effect (Wang *et al.*, 2014). Moreover, the signal transduction pathways involved in response to aphid and caterpillar feeding are found to cross-talk and could work antagonistically depending on the order of herbivore arrival (Pieterse *et al.*, 2009; Thaler *et al.*, 2012; Wei *et al.*, 2014). Therefore, we expect that the timing and order of arrival of herbivores will interact particularly in relation to plant responses to herbivores of different feeding guilds and thus with regard to shaping the plant phenotype experienced by subsequent herbivores in the interaction network.

Here, we investigated whether the induced plant phenotype in response to the order and timing of arrival of two herbivores affected the preference and performance of a subsequently arriving third herbivore. We conducted two-choice feeding preference tests with the generalist caterpillar *Mamestra brassicae* L. (Lepidoptera: Noctuidae) and examined *M. brassicae* weight gain in performance tests on *Brassica oleracea* L. (Brassicaceae) plants previously infested with *Brevicoryne brassicae* L. (Hemiptera: Aphididae) aphids and/or *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) caterpillars. All herbivores used in the experiments are known to feed on wild cabbage plants under natural conditions (Moyes *et al.*, 2000; Newton *et al.*, 2009). Both *B. brassicae* and *P. xylostella* arrive at similar moments early in the growth season of *B. oleracea* plants (Poelman *et al.*, 2009, 2010), which results in variation in the arrival pattern of the two herbivores on individual plants. The plants may be colonised initially by either *B. brassicae* aphids or *P. xylostella* caterpillars, with a varying arrival time between the arrival of the two herbivores on a single plant (Poelman *et al.*, 2009, 2010). Subsequently, *M. brassicae* caterpillars that hatch from eggs in these plant–insect communities disperse and search for suitable food plants (Goulson & Cory, 1995). These herbivores are thus exposed to phenotypic variation in *B. oleracea* induced by sequential feeding by aphids or caterpillars (Gols *et al.*, 2008a; Soler *et al.*, 2012; Li *et al.*, 2014; Stam *et al.*, unpublished). Our experiments tested the hypothesis that the order and timing of arrival of two herbivore inducers interact in their effect on food-plant choice and performance of a subsequently feeding herbivore. It was expected that time and order of arrival would interact, as the nature and time course of induced plant responses to each herbivore are likely to differ and thereby sequential induction by different species would further differentially modulate plant responses (Thaler *et al.*, 2002; Voelckel & Baldwin, 2004). For example, when whiteflies arrived before powdery mildew on tomato plants, this had a negative effect on the latter attacker only with a short arrival interval between attackers whereas when the powdery mildew arrived before the whiteflies, this had a positive effect on the latter attacker only with a long arrival interval between attackers (Mouttet *et al.*, 2013). The interaction between timing and

order of arrival may thus have an impact on food plant preference and performance of newly arriving herbivores. We hypothesised that the performance of *M. brassicae* would be worse on plants that had undergone double versus single herbivore attack, and would also be poorer when plants had experienced initial feeding and longer induction by the caterpillars of *P. xylostella* rather than aphids. Because aphids are known to dampen the plant-induced response by caterpillars, caterpillars of *M. brassicae* were expected to perform better on plants that were initially induced by aphids and where there was a longer period before the subsequent arrival of *P. xylostella* caterpillars. Because of the expected poorer performance on plants that had received caterpillar damage first, we hypothesised that *M. brassicae* caterpillars would avoid these plants for feeding in choice situations with plants that were induced by aphids first. We discuss the implications of our results for plant-mediated interactions among multiple herbivores and provide future directions on ecological and evolutionary studies of plant–insect interactions.

Materials and methods

Plants and insects

Seeds of wild *Brassica oleracea* from a population in Kimmeridge, Dorset, UK (50°36'N, 2°07'W; Gols *et al.*, 2008b) were used in all experiments. Seeds were germinated on humid potting soil (Lentse potgrond, Lent, the Netherlands) and kept cool (4 °C) overnight, followed by 1 week under greenhouse conditions (22 ± 5 °C, RH 50–70%, LD 16:8 h). In the same greenhouse, seedlings were transplanted to 1.45-litre pots containing potting soil. Plants were watered daily and used for experiments when 3 weeks old.

Specialist herbivores, i.e., the caterpillars *Plutella xylostella* and aphids *Brevicoryne brassicae*, as well as generalist caterpillars *Mamestra brassicae* were reared at the Laboratory of Entomology, Wageningen University, the Netherlands. The insects were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv. *Cyrus*) under greenhouse conditions (21 ± 2 °C, RH 50–70%, L:D 16:8 h). All experiments were conducted under greenhouse conditions (22 ± 5 °C, RH 50–70%, L:D 16:8 h), in two subsequent blocks in early and mid-April 2013.

Plant phenotype induction by and performance of the herbivores *Brevicoryne brassicae* and *Plutella xylostella*

To test whether the order and timing of arrival of two herbivores affected preference and performance of a third herbivore *M. brassicae*, we induced wild *B. oleracea* plants with different patterns of attack by either 15 wingless adult *B. brassicae* aphids and/or six second larval stage (L2) *P. xylostella* caterpillars (Fig. 1a). Each species was equally divided over three fully unfolded leaves per plant (Fig. 1b). During the induction period, plants were individually covered with a fine gauze net to avoid cross-contamination of insects among plants. Because induction treatments differed in duration to allow for variation in order and timing of herbivore induction but should be ready

at the same time to allow direct comparison of their effect on preference and performance of the third herbivore *M. brassicae*, we aligned all treatments to the end point of induction (Fig. 1a). In total, 12 treatments were prepared, including undamaged plants, plants induced with a single herbivore species (six treatments) and double herbivore treatments in which two herbivore species were inoculated with different order and timing of arrival (five treatments).

The undamaged plants (N) served as controls to measure the direction and strength of effect by single and double herbivore treatments on *M. brassicae* preference and performance. To establish a baseline of how the aphid *B. brassicae* (A) and caterpillar *P. xylostella* (C) individually affected preference and performance of *M. brassicae*, we induced plants with a single herbivore species. Treatments in which each herbivore species was feeding alone were prepared with different duration of herbivore induction to function as controls for duration of induction by that herbivore in double herbivore-induced plants. Aphids were inoculated and allowed to feed for 5 (A), 4 (oA), or 2 days (ooA) before plants were included in experiments with *M. brassicae*. Similar treatments were prepared for caterpillar feeding (C, oC, ooC), in which (o) indicates a day without herbivore inoculation. Double herbivore treatments included a treatment in which both herbivores were inoculated at the same time and were allowed to feed for 5 days (B). Four treatments differed in the order of herbivore arrival (aphids or caterpillars first) and consisted of subsequent arrival of the second herbivore after 1 day (AC, CA) or 3 days (AooC, CooA). Also in these treatments the first herbivore fed on the plants for 5 days. After this, *M. brassicae* preference and performance tests were conducted (see later).

The presence, order, and timing of arrival of inducing herbivores could affect the performance of the inducing herbivores, thereby possibly affecting the strength of induction among treatments. Therefore, the performance of both inducing herbivores was assessed to help explain the preference and performance of the subsequently feeding *M. brassicae* caterpillars. Performance of *B. brassicae* was assessed by counting the total number of aphids (adults + offspring) per plant at the end of the induction period. The performance of *P. xylostella* was assessed by weighing each individual caterpillar at the end of the induction period with a microbalance (Sartorius CP2P, Sartorius AG, Goettingen, Germany; accuracy 0.001 mg). Numbers of replicates for both herbivore species varied among treatments, because some plants with accidental mechanical damage were eliminated from further experiments and analyses.

Mamestra brassicae caterpillar feeding choice test

In order to test the hypothesis that *M. brassicae* caterpillar feeding preference is affected by the combination of timing and order of previously arriving herbivores, we used one set of induced plants (described earlier) to conduct a series of two-choice tests (Fig. 1). We focused on three sub-questions. First, we tested whether *M. brassicae* caterpillars had a preference for aphid (A)- or caterpillar (C)-induced plants over undamaged plants (N). Second, to test whether timing and order of arrival between herbivore inducers affected *M. brassicae*

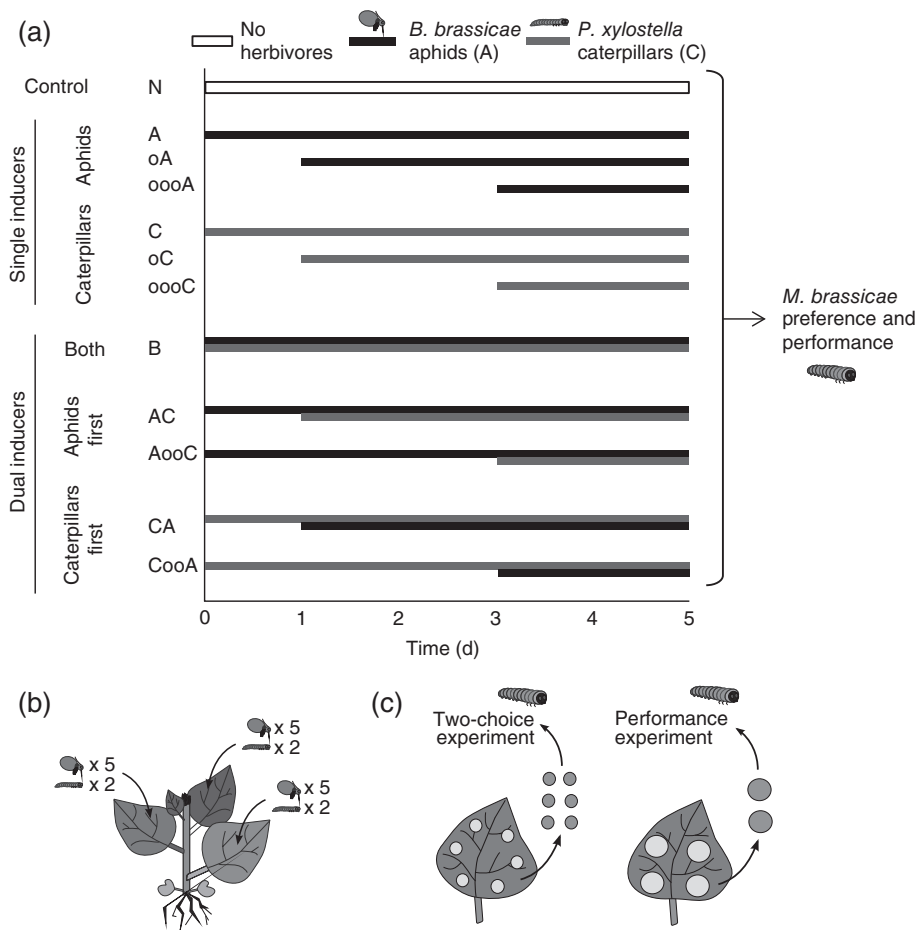


Fig. 1. Overview of experimental setup, testing preference, and performance of herbivores arriving in different orders and with different times of arrival on *Brassica oleracea*. (a) Twelve treatments applied to the plants. Treatment capital letters indicate the start of plant induction on day 0: N, no herbivores; A, aphids, *Brevicoryne brassicae*; C, caterpillars, *Plutella xylostella*; B, both insects simultaneously arriving on day 0; o, no new herbivore induction initiated on that day. Performance of the two inducing herbivores is measured after day 5. *Mamestra brassicae* preference and performance on those induced plants is subsequently tested in a 24-h choice test, and after a 48-h growth bioassay, respectively. Only a subset of plants are used for the *M. brassicae* choice test, using only comparisons among treatments that differed most in timing of arrival between inducers (N, A, C, B, CooA, AooC), omitting treatments with 1-d interval between arrival of the two herbivores and their controls (AC, CA, oC, oA). (b) The infestation procedure. The three youngest unfolded leaves were infested with five aphids and/or two caterpillars according to the treatments. (c) Sampling of leaf disks. After the induction period, leaf disks were randomly sampled from each infested leaf. For the two-choice experiment (left), six leaf disks were sampled; for the performance experiment (right), two leaf disks were sampled per induced leaf.

food-plant preference, we tested the preference for undamaged (N) versus double herbivore-damaged plants and used only the treatments in which the herbivores were introduced at the most widely differing arrival times: simultaneous (B), or with a 3-day delay of the second herbivore inoculation for treatments in which aphids (AooC) or caterpillars were inoculated first (CooA). We complemented this with a direct comparison between herbivore order of arrival (CooA versus AooC). Third, we paired leaf disks from plants induced by aphids (A) or caterpillars (C) alone, or both herbivores simultaneously (B) with all other treatments (N, A, C, B, CooA, AooC) to gain a detailed insight into caterpillar preference for treatments with single and double herbivore induction.

Each of the two-choice tests was set up as follows. At the end of the induction period described earlier, all herbivores

were removed and we took six leaf disks from each of the three induced leaves per plant (diameter 1.6 cm; total 18 disks per plant), avoiding visible herbivore damage and the main vein (Fig. 1c). Using leaf disks allowed us to test a large number of treatment combinations, to exclude visual damage driving herbivore choices and to homogenise leaf shape, even though the leaf disks might have been affected in quality by excision of the disks. The latter, however, affected samples from all treatments. Leaf disks from different plant individuals within the same treatment were randomly combined with leaf disks from other treatments. In a 5.5-cm Petri dish lined with moist filter paper, two leaf disks from different treatments were placed on opposite sides, and one newly hatched (L1) *M. brassicae* caterpillar was introduced in the middle. Caterpillars were allowed to make a choice by feeding on the two leaf disks for

24 h under ambient room temperature conditions. After visual inspection for area of leaf damage, the leaf disk that had greatest caterpillar feeding damage was considered to be the preferred treatment. When no difference in consumed leaf area between the two disks could be detected, the replicate was scored as 'no choice'. Each caterpillar was a replication unit, with 51 replicates for each of the choice combinations.

Mamestra brassicae caterpillar performance

To test the hypothesis that *M. brassicae* caterpillar performance is influenced by previous induction by herbivores arriving with differing order and timing, we set up a feeding performance experiment. Another set of plants previously induced by *B. brassicae* and/or *P. xylostella* was used to assess *M. brassicae* performance when subsequently feeding on those plants (Fig. 1). All herbivore induction treatments described earlier were offered to *M. brassicae* caterpillars.

At the end of the induction period, all herbivores were removed and two leaf disks were taken (diameter 2.4 cm; total of six disks per plant) from each of the three induced leaves per plant, avoiding visible herbivore damage and the main vein (Fig. 1c). Each leaf disk was individually placed in a 5.5-cm Petri dish lined with moist filter paper. Per dish, one L2–L3 *M. brassicae* caterpillar was carefully introduced, after it had been weighed on an analytical balance (ML 4/01, accuracy 0.1 mg; Mettler Toledo, Greifensee, Switzerland). Caterpillars were allowed to feed on the leaf disk for 48 h under greenhouse conditions. Their feeding and growth were stopped by storing them at 4 °C until re-weighing 13 days later with the same balance. Pilot tests demonstrated that storage did not affect caterpillar weight (data not shown). Absolute growth of *M. brassicae* during the 48-h feeding period was then calculated as (weight after – weight before). Individual caterpillars were a replication unit, with 39–45 replicates per induction treatment.

Statistical analyses

To compare performance of the inducing herbivores across treatments with different feeding durations, daily increase in aphid population per plant and daily caterpillar growth were calculated by dividing the total number of aphids or caterpillar weight by the number of days they spent feeding on the plant. Daily increase in aphid numbers was \log_{10} -transformed to meet test assumptions. The effects of herbivory treatment, block, and their interaction were tested with two-way ANOVA, followed by least significant difference (LSD) *post hoc* tests if results were significant. To meet test assumptions, caterpillar weights were double log-transformed $\{x' = \log[-\log(x/100)]\}$ prior to analysis on effects of herbivore treatment, block and their interaction with a linear mixed model (LMM). Treatment and block were used as main factors and plant identity as random factor, followed by an LSD *post hoc* test if results were significant.

To analyse *M. brassicae* preference for plants induced by our different herbivore treatments, we compared whether the proportion of *M. brassicae* caterpillars choosing for either of the inducing-herbivore treatments differed from a 50:50 ratio using

a binomial exact test. Second, we tested differences in choice distributions between different treatment combinations with a generalised linear model (GLM) with binomial distribution and logit link function. Either herbivore treatment, the block in which the experiment was conducted, or the interaction between the two were included in the model to obtain Wald statistics for GLM tests for each of the factors and their interaction. Choice distributions were tested among leaf disks of the full-factorial set of induction treatments versus leaf disks of undamaged, aphid-, caterpillar-, or aphid- and caterpillar-damaged plants. Choices in which treatments were paired with leaf disks of undamaged plants were split into two analyses: undamaged (N) versus single herbivore species (A, C) or two herbivores (B, AooC, CooA). Finally, differences in the numbers of 'no choice' between treatments, blocks or their interaction were similarly analysed with a GLM.

To test the effect of induction treatments on *M. brassicae* performance, we first square-root-transformed *M. brassicae* caterpillar weight-increase values to meet test assumptions on homogeneity and normality. We first tested overall effects of herbivory treatment, experimental block and their interaction on *M. brassicae* performance using an LMM. Treatment and block were used as main factors and plant identity as random factor. For this overall test, treatments were categorised in four groups, consisting of no herbivory (N); insect identity [aphid only (A, oA, oooA) or caterpillars only (C, oC, oooC)]; and both herbivores present (B, AC, CA, AooC, CooA). Then, effects of the identity of single inducing species [aphids (A, oA, oooA) or caterpillars (C, oC, oooC)], the time spent on the plant, the experimental block and their interactions were similarly analysed with an LMM including plant identity as random factor. Finally, we analysed effects of inducing species that arrived first on plants, timing of inoculation of the second herbivore, experimental block and their interactions in similar models, limiting the dataset to treatments in which both inducers were present on the plant (B, AC, CA, AooC, CooA). In the latter two analyses, the no herbivory treatment (N) was not included as this had no factor of either species or feeding duration/timing as specified in each LLM. Furthermore, *M. brassicae* is likely to arrive on plants that have undergone previous herbivory rather than on completely undamaged plants (Poelman *et al.*, 2009, 2010; Stam *et al.*, unpublished) and we therefore focused our *post hoc* analyses depicted in Fig. 5 on the statistical models that compared single and double herbivore treatments. Linear mixed models on *M. brassicae* and *P. xylostella* performance, and binomial exact test and GLM on *M. brassicae* choice and no-choice were conducted with GENSTAT software version 17.1 (VSN International, Hemel Hempstead, UK). An ANOVA was conducted with SPSS version 22.0.0.1 to analyse *B. brassicae* performance (IBM Corp., Armonk, New York).

Results

Performance of inducing herbivores *Brevicoryne brassicae* and *Plutella xylostella*

During the induction period, the performance of *B. brassicae* aphids and *P. xylostella* caterpillars was affected by the induction

Table 1. General linear (mixed) model for herbivore performance.

Insect performance	Treatment			Block			Treatment × block		
	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>	d.f.	<i>F</i>	<i>P</i>
<i>Brevicoryne brassicae</i> performance	7	3.324	0.003	1	118.062	< 0.001	7	0.846	0.552
<i>Plutella xylostella</i> performance	7	18.20	< 0.001	1	73.24	< 0.001	7	2.92	0.007
<i>Mamestra brassicae</i> performance; overall	3	1.72	0.166	1	143.37	< 0.001	3	0.40	0.754

Difference in performance of the inducers *B. brassicae* and *P. xylostella* on plants induced by herbivory treatments, blocks, and their interaction. Differences in overall *M. brassicae* performance after feeding on plants induced by different herbivory treatments, blocks, and their interaction. *Mamestra brassicae* and *P. xylostella*: linear mixed model, with plant identity as random factor included in the model; *B. brassicae*: ANOVA. *Mamestra brassicae* performance was tested for overall effects of herbivory treatments: no herbivory (N); insect identity: aphid only (A, oA, oooA), caterpillars only (C, oC, oooC); and both herbivores present (B, AC, CA, AooC, CooA). Numbers in bold indicate significant effects ($\alpha = 0.05$).

treatment in which the herbivores were either feeding alone or together (Table 1). When the aphid *B. brassicae* was inoculated on the plant together with *P. xylostella*, the increase in the number of aphids was positively affected by a longer arrival time between inoculation of the two herbivores, as compared with simultaneous arrival, but only when caterpillars had been inoculated first (Fig. 2). For *P. xylostella* caterpillar performance, the order of inoculation resulted in a different direction of effect of timing between inoculation of the two herbivores. When caterpillars were inoculated first, *P. xylostella* caterpillar growth increased with increasing time between herbivore arrivals compared to simultaneous arrival, whereas with aphids arriving first, caterpillar growth decreased with increasing time between herbivore inoculation events compared to simultaneous arrival. The latter effect may be a result of the lower daily growth rate of caterpillars that were feeding for a smaller number of days, visible when they were feeding alone (Fig. 3). Thus, for both of the inducing herbivores, an interaction between order and timing of arrival seems to occur, as both factors together determine the resulting performance of the inducers.

Mamestra brassicae caterpillar preference

In pairwise food plant preference tests, the number of non-responding *M. brassicae* caterpillars (11–32%; Fig. 4) was not affected by the different choice combinations that were offered to the caterpillars (Table 2), indicating that *M. brassicae* choice behaviour was consistent over the experiments. The choice of *M. brassicae* was not strongly affected by previous herbivory on the leaves, as most of the preference tests did not yield significant results. However, *M. brassicae* caterpillars preferred to feed on leaf material of undamaged plants than on plants infested simultaneously by aphids and caterpillars (N versus B; Fig. 4). In all other pairwise choices between undamaged versus herbivore-induced plant treatments, and aphid-, caterpillar- and double herbivore-induced versus all other treatments in the full-factorial design, *M. brassicae* did not show a feeding preference for one of the treatments (Fig. 4).

However, the distribution of *M. brassicae* choices among the herbivore–treatment combinations that were offered revealed some differences (Table 2). Although *M. brassicae* did not choose differently for either aphids alone or caterpillars alone over undamaged leaves (Fig. 4a), choices for undamaged

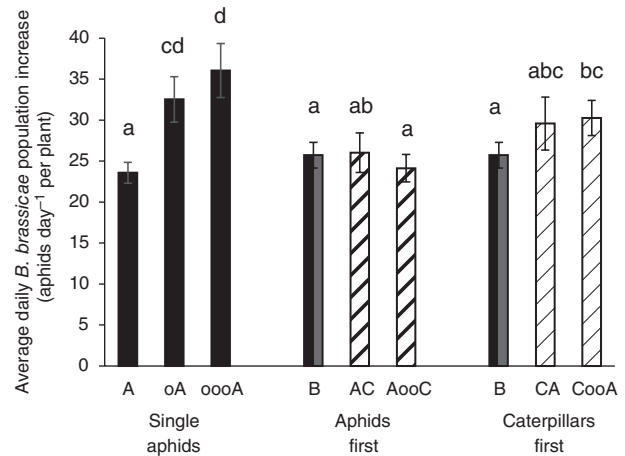


Fig. 2. *Brevicoryne brassicae* aphid performance when feeding during different periods before, after, or together with *Plutella xylostella* caterpillars. Daily aphid population increase per plant (\pm SE). Treatment capital letters indicate time of induction on the plant, starting from day 0: B, both insects arriving simultaneously on day 0; A, aphids; C, caterpillars; o, no new herbivore induction initiated on that day. Treatment 'B' is shown twice for clarity of comparisons. Different lower-case letters indicate groups that differ significantly.

versus damaged leaves by both herbivores differed significantly depending on the order and timing of arrival of those herbivores (Table 2; Fig. 4b). *Mamestra brassicae* more frequently consumed a larger leaf area from plants in which aphids were introduced first followed by caterpillars (AooC) than from undamaged plants in paired choice tests, whereas plants induced by caterpillars and subsequently by aphids (CooA), or plants that were induced by both herbivores simultaneously (B), were avoided in preference for undamaged plants (lower-case letters in Fig. 4b; LSD comparisons: N/AooC versus N/CooA, $P = 0.025$; N/AooC versus N/B, $P = 0.004$; N/B versus N/CooA, $P = 0.478$). In line with this observation, when comparing only between the different orders of herbivore arrival [caterpillars first (CooA) versus aphids first (AooC)], *M. brassicae* preference was similar to the comparisons against undamaged leaves. *Mamestra brassicae* caterpillars more frequently consumed a larger area from plants that were first induced by aphids followed by caterpillars (AooC) than from plants induced in the reverse order (CooA), although this

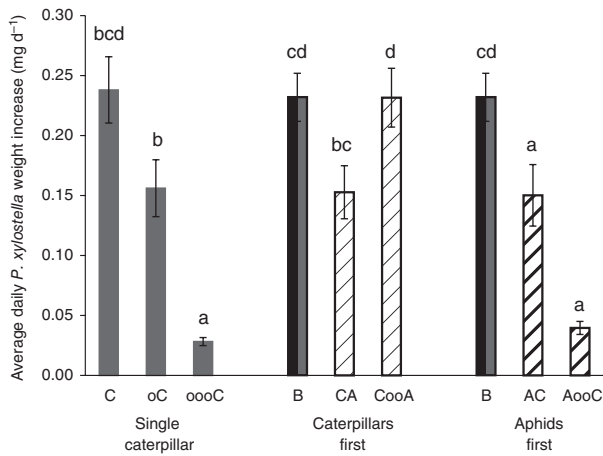


Fig. 3. *Plutella xylostella* caterpillar performance when feeding during different periods before, after, or together with *Brevicoryne brassicae* aphids. Average increase of caterpillar weight per day, untransformed data (\pm SE). Treatment capital letters indicate induction treatments by aphids *B. brassicae* and/or caterpillars *P. xylostella*: 5 (C), 4 (oC) or 2 (oooC) days of herbivory by C (caterpillars); B, 5 days' herbivory by both insects simultaneously, and four treatments of 5 days' herbivory by aphids and caterpillars in which aphids were inoculated first, followed by caterpillars with 1 (AC) or 3 days (AooC) between arrivals, or in the reverse order (CA, CooA). Treatment B is shown twice for clarity of comparisons. Different lower-case letters indicate significantly different groups. Statistical analysis of these data was conducted on double-log-transformed data to meet test assumptions, hence the *post hoc* lower-case letters should not be extrapolated based on the current figure presenting the untransformed data.

comparison was not significantly different from a 50:50 ratio (Fig. 4b). In choice tests between leaf material of plants induced by single herbivores [aphid (A) or caterpillar (C)] or both herbivores simultaneously (B) versus all other treatments in the factorial design, the distribution of *M. brassicae* choices did not vary significantly between any of the two-choice combinations (Fig. 4c; Table 2).

Mamestra brassicae caterpillar performance

In an overall comparison of the performance of *M. brassicae* caterpillars in terms of weight gain on plants treated with no previous inducers, only aphids, only caterpillars or both inducers, no significant differences were recorded (Table 1). However, when comparing only plants with single inducers, *M. brassicae* caterpillar performance was affected by the species of inducer (Table 3). *Mamestra brassicae* had, on average, a 12% higher weight increase when feeding on aphid-induced plants (average of A, oA and oooA) than when feeding on caterpillar-induced plants (average of C, oC and oooC; Fig. 5a). For both single caterpillar and single aphid infestations, the duration of prior induction of 2, 4, or 5 days did not significantly affect the performance of subsequently arriving *M. brassicae* caterpillars (Table 3). However, the timing of induction was important for the performance of *M. brassicae* when feeding on plants induced by both caterpillars and aphids (Table 3).

Pairwise comparisons show that *M. brassicae* gained more weight on plants exposed to dual infestation when the time between infestations with aphids and caterpillars was long (3 days) than when the time between infestations was short (1 or 0 days; Fig. 5b). More specifically, *M. brassicae* grew on average 28% more on plants with a 3-day period between arrival of inducing herbivores (average of AooC and CooA) than on those with a 0-day period (B; LMM: $Wald_1:6.84$, $P = 0.009$), and 19% more compared with a 1-day period (average of AC and CA; LMM: $Wald_1:4.28$, $P = 0.047$); while the 7% increased growth on plants with 1 day between arrival of inducers (AC and CA) was not significantly different from the performance on plants with simultaneous infestation of both herbivores (B; LMM: $Wald_1:0.69$, $P = 0.406$). The order of herbivore infestation on the plants (aphids first or caterpillars first) did not affect the performance of *M. brassicae* (Table 3). Also no interaction was found between order and timing of arrival of the two herbivores with regard to the growth of subsequent feeding *M. brassicae* caterpillars (Table 3).

Discussion

Plant-mediated interactions between herbivores have a dynamic character in nature. The consequences of the dynamics between two herbivores for other community members due to variation in the timing of herbivore colonisation on an individual plant are still poorly understood (Gomez *et al.*, 2010; Karban, 2011; Underwood, 2012). We found that interactions between the aphid *B. brassicae* and *P. xylostella* caterpillars were affected by their order and the arrival time between inoculations and that these factors in a double herbivore attack had minor effects on preference and performance of caterpillars of *M. brassicae* as a third herbivore interacting with the induced plant. Overall, the order of herbivore arrival in a double herbivore attack had opposite effects on food plant preference by the third herbivore *M. brassicae*, which preferred plants damaged by aphids first to undamaged plants, and preferred undamaged plants to those damaged by caterpillars first. The timing of arrival between herbivores did not strongly influence the preference of *M. brassicae* caterpillars. However, *M. brassicae* weight increase was larger, the longer the time between inoculation of the two inducers. This was not due to the difference in the time the inducers spent feeding on the plant, as *M. brassicae* did not experience a growth difference between plants with various feeding durations of aphids alone and caterpillars alone. The feeding preference and performance of *M. brassicae* were not affected by the interaction of the order and timing of previous feeding by two herbivores. In the network of interactions among the three herbivores, the performance of the first two herbivores in the network did not directly explain feeding preference or performance of the third herbivore *M. brassicae*.

Identity and order of arrival of inducing herbivores

Mamestra brassicae caterpillars grew faster on aphid-induced than on caterpillar-induced plants (Fig. 5a). This pattern fits in the pattern of differential induction of phytohormonal pathways by herbivores from different feeding guilds (Thaler *et al.*,

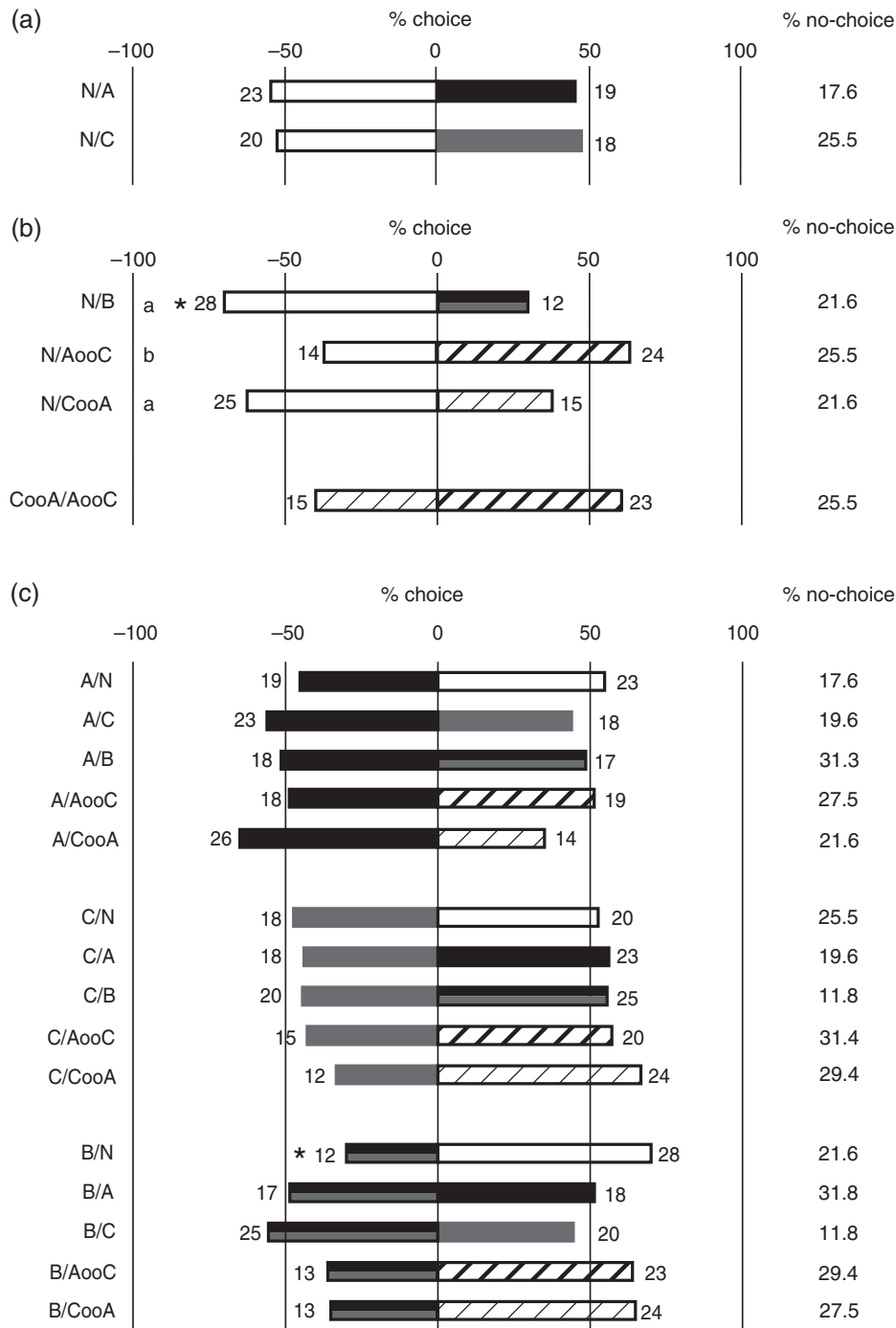


Fig. 4. *Mamestra brassicae* caterpillar preference for plants previously induced by different order and timing of arrival of two herbivores. Bars show the percentage of *M. brassicae* choices for either of two treatments in a two-choice test, and next to each bar are the absolute numbers of *M. brassicae* caterpillars that chose for a treatment. Capital letters indicate the treatment combination in the choice test: N, no herbivory; A, 5 days of herbivory by aphids; C, 5 days of herbivory by caterpillars; B, both insects simultaneously and two treatments of 5 days' herbivory by aphids and caterpillars in which aphids were inoculated first followed by caterpillars on day 3 (AooC) or in reverse order (CooA). (a) Choice tests of induction by single herbivore species compared with undamaged plants; (b) choice tests of dual-inducing herbivores compared with undamaged plants; (c) choice tests in which the treatments A, C, and B were paired with all other treatments. Some choice combinations are shown repeatedly for clarity of comparisons. Asterisks (*) indicate choices that differed significantly from 50:50 choice ratio, while different lower-case letters indicate choice combinations that differ significantly among each other in a generalised linear model (GLM) analysis. In (b), choice combination 'CooA/AooC' was not included in the GLM test for different choice distributions among choice combinations (versus undamaged plants). The column on the right indicates the percentage of *M. brassicae* that did not make a choice (% no-choice).

Table 2. Generalised linear model (GLM) Wald table for *Mamestra brassicae* caterpillar choice distributions.

Preference combinations tested	Preference			Block			Preference × block		
	d.f.	Wald	P	d.f.	Wald	P	d.f.	Wald	P
Single herbivores versus N	1	0.036	0.849	1	0.007	0.932	1	0.202	0.653
Dual herbivores versus N	2	9.138	0.010	1	0.158	0.691	2	6.034	0.049
All treatments versus A	4	3.749	0.441	1	9.715	0.002	4	6.122	0.190
All treatments versus C	4	1.692	0.792	1	3.621	0.057	4	3.337	0.503
All treatments versus B	4	7.455	0.114	1	0.002	0.969	4	4.846	0.304
No-choice (overall)	14	11.22	0.668	1	3.015	0.083	29	20.67	0.871

Differences in *M. brassicae* choice distributions and no-choice distributions between preference tests, blocks, or their interaction. Preference tests were two-treatment combinations in a full factorial design of herbivore-induced plants against undamaged plants (N), aphid-damaged plants (A), caterpillar-damaged plants (C) or plants damaged by both herbivores (B). GLM tests against undamaged plants were split into 'single herbivores against N' (N/A, N/C) and 'dual herbivores against N' (N/B, N/AooC, N/CooA). Wald indicates Wald statistic, and P gives χ^2 probability. Numbers in bold indicate significant effects ($\alpha = 0.05$).

Table 3. Generalised linear mixed model for *Mamestra brassicae* performance on single or double species-induced plants.

<i>M. brassicae</i> performance	Time (1)			Species (2)			Block (3)					
	d.f.	F	P	d.f.	F	P	d.f.	F	P			
<i>M. brassicae</i> performance; single herbivory	2	0.56	0.573	1	4.49	0.035	1	91.43	< 0.001			
<i>M. brassicae</i> performance; double herbivory	2	3.93	0.028	1	2.46	0.125	1	37.54	< 0.001			
	1 × 2			1 × 3			2 × 3			1 × 2 × 3		
	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
<i>M. brassicae</i> performance; single herbivory	2	0.12	0.890	2	1.41	0.247	1	0.04	0.839	2	1.64	0.196
<i>M. brassicae</i> performance; double herbivory	1	0.48	0.491	2	0.75	0.482	1	0.86	0.359	1	1.75	0.195

Difference in *M. brassicae* performance after feeding on plants induced by different herbivory treatments: linear mixed model, with plant identity as random factor included in the model. Factors were tested as follows: when *M. brassicae* was feeding on plants with single herbivores only (A, oA, oooA, C, oC, oooC), effects of time herbivores spent on the plant (5d, 4d or 2d), species (aphids or caterpillars), blocks, and all interactions were tested; when *M. brassicae* was feeding on plants with double herbivory only (B, AC, AooC, CA, CooA), effects of arrival time between herbivore arrivals (0, 1, or 3 d), species order of arrival (aphids first or caterpillars first), blocks, and all interactions were tested. Numbers in bold indicate significant effects ($\alpha = 0.05$).

2002; Voelckel & Baldwin, 2004; de Vos *et al.*, 2005; Ali & Agrawal, 2014). The phytohormonal pathway generally induced by phloem feeders such as aphids may negatively interfere with the phytohormonal pathway induced by caterpillars, thus alleviating plant resistance against caterpillars (Zarate *et al.*, 2007; Soler *et al.*, 2012; Zhang *et al.*, 2013).

Here, it was not only the identities of the inducing herbivore species that were important for subsequent feeding choice of *M. brassicae*, but also the order in which they infested the plant (Fig. 4). Our data on *M. brassica* choice are in line with the pattern in the literature on herbivore responses (preference, but also the resulting performance) to induced plants (Thaler *et al.*, 2002; Zhang *et al.*, 2009; Soler *et al.*, 2012; Mathur *et al.*, 2013): *M. brassicae* preferred plants on which aphids had arrived first to undamaged plants (AooC/N), but avoided plants on which another caterpillar species had arrived first in preference for undamaged plants (CooA/N), regardless of simultaneous or subsequent induction by the other herbivore (Fig. 4b). Even though we did not find effects of the arrival order on the performance of *M. brassicae* (Table 3), the importance of herbivore order of arrival for herbivore performance has previously been identified in pairwise interactions among

above- and below-ground herbivores (Erb *et al.*, 2011; Wang *et al.*, 2014). These studies identified that the first-arriving herbivore had negative effects on the second entrant, but the second herbivore had neutral effects on the performance of the first herbivore (Erb *et al.*, 2011; Wang *et al.*, 2014). Beyond these pairwise interactions, the order of herbivore arrival has also been found to affect the occurrence of subsequent community members later in the season (Viswanathan *et al.*, 2007; Miller-Pierce & Preisser, 2012; Stam *et al.*, unpublished). For example, on *Solanum dulcamara* plants, the first-arriving insect always determined the numbers of herbivores on that plant later in the season (Viswanathan *et al.*, 2007). This could indicate that the plant prioritises its responses to the first-arriving herbivore (Viswanathan *et al.*, 2007; Miller-Pierce & Preisser, 2012; Stam *et al.*, 2014). However, studies focusing on plant responses by measuring transcriptome responses to double-stress suggest that the second inducer at least leaves a strong signature on plant responses shortly after feeding (Voelckel & Baldwin, 2004; Coolen *et al.*, 2016; Davila Olivares *et al.*, 2016). It remains to be identified whether these translate into strong phenotypic changes or whether this discrepancy in the literature is caused by specificity of double-stress in different plant species. In addition

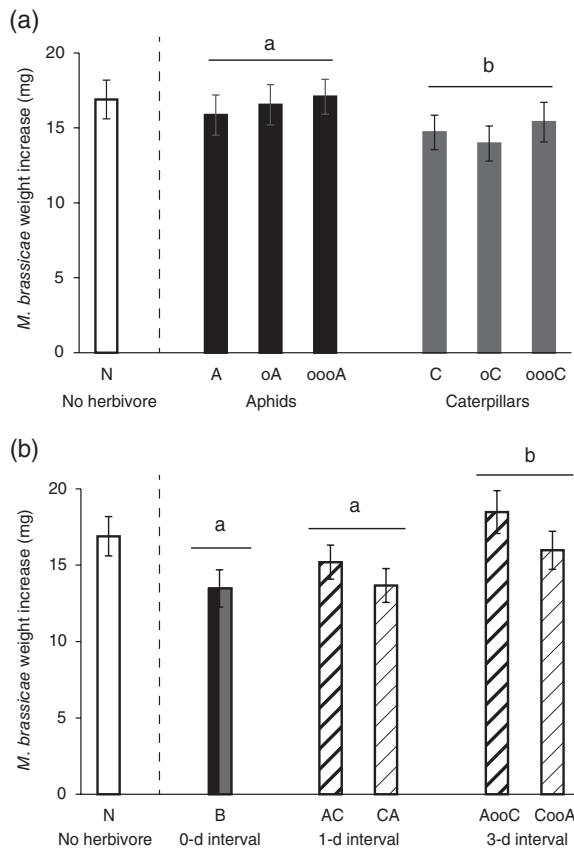


Fig. 5. *Mamestra brassicae* caterpillar performance when feeding on plants previously induced by different order and timing of arrival of two herbivores. Average weight increase (weight after – weight before) (\pm SE). Treatment capital letters indicate induction treatments by aphids, *Brevicoryne brassicae*, and/or caterpillars, *Plutella xylostella*; N, no previous herbivory; A, 5 days of herbivory by aphids; C, 5 days of herbivory by caterpillars; B, both insects simultaneously and four treatments of 5 days' herbivory by aphids and caterpillars in which aphids were inoculated first followed by caterpillars with 1 (AC) or 3 days (AooC) between arrivals, or in the reverse order (CA, CooA). (a) *Mamestra brassicae* performance on plants previously induced with single herbivores; (b) *M. brassicae* performance on plants previously induced with both herbivores. Different lower-case letters indicate groups that differ significantly in statistical analyses for effect of herbivore inducer and timing of arrival between herbivores. For comparison, treatment 'N' is included in the graphs, although this treatment is only included in statistical models that tested overall treatment effects (Table 1).

to the effects of herbivore order of arrival, our data show that the performance of a third herbivore may also be influenced by the timing of arrival of herbivores in double herbivory treatments.

Time of arrival of inducing herbivores

Mamestra brassicae caterpillars performed better on plants that were induced by double herbivore attack with a longer arrival time between infestation by the two herbivores as compared with a shorter arrival time (Fig. 5b). This contrasts to a

study on *Plantago lanceolata* in which a longer time between the arrival of conspecifics caused a decrease in the consumption of leaf area by the second-arriving *Spodoptera exigua* caterpillars (Wang *et al.*, 2015). However, in another study with *S. exigua* caterpillars attacking tomato plants twice, caterpillar bioassays after a short or longer period following the two attacks showed that a short arrival time after repeated attack yielded a lower plant resistance response than that after the first attack only (Underwood, 2012). In contrast, no decrease in plant resistance was observed with a longer time between repeated herbivore attack and the bioassay (Underwood, 2012). This indicates that a response to a first herbivore attack limits plants in the strength and speed of a response to a second attacker depending on the timing of each subsequent attack (Karban, 2011; Underwood, 2012).

Alternatively, when insects from different feeding guilds arrive in sequence, other interactions of plant responses to a first and second herbivore might occur as compared with subsequent attack by conspecifics. When two herbivores from different guilds arrive simultaneously or shortly after one another, they could arrive within the time lag of a herbivore feeding guild-specific plant response that occurs after the first herbivore starts feeding (Gomez *et al.*, 2010; Karban, 2011). In that case, the herbivore-specific plant response to both herbivores simultaneously may interact or add up to produce a stronger resistance response than a plant response to single herbivory (Voelckel & Baldwin, 2004; Johnson *et al.*, 2006). Even synergistic responses might occur in which the resulting plant resistance against herbivores is higher than the sum of resistances against each of the single herbivores (Pieterse *et al.*, 2009; Menzel *et al.*, 2014). Especially with plant responses to different feeding guilds, cross-talk between plant responses could cause such synergistic effects (Xu *et al.*, 1994; Zhang *et al.*, 2013). On the other hand, with a longer arrival time between arrival of two herbivore species, the second herbivore may arrive when the plant has already started responding to the first herbivore (Karban, 2011). In that case, plant responses could be delayed or prolonged, but with limitations in strength and speed of the resistance response that a plant can mount to a second attacker (Underwood, 1998, 2012; Karban, 2011; Thaler *et al.*, 2012). Also non-additive effects could occur if plant responses to different feeding guilds show antagonistic interference within this arrival time (Thaler *et al.*, 2002; Zhang *et al.*, 2009; Soler *et al.*, 2012). This could result in lower resistance responses and would explain a better performance of caterpillars on a plant phenotype induced by two herbivores with a long arrival time between their arrival, as was found for *M. brassicae* in our study.

As plant responses to herbivores from different feeding guilds are often asymmetric (Kaplan & Denno, 2007), and we observed that the timing of herbivore arrival affects the performance of a subsequent herbivore (Wang *et al.*, 2015), we expected an interaction between the effects of order and timing of inducer arrival on *M. brassicae* performance. Feeding by the aphid *B. brassicae* was expected to dampen the effect of *P. xylostella* induction, because of antagonistic effects of plant physiological responses of aphids to responses induced by caterpillars (Thaler *et al.*, 2002; Voelckel & Baldwin, 2004; Stam *et al.*, 2014). These effects were hypothesised to be stronger the longer

aphids were established before the arrival of *P. xylostella* caterpillars, because plants would in these situations have mounted stronger physiological responses to aphids (e.g. see Viswanathan *et al.*, 2007; Miller-Pierce & Preisser, 2012; Stam *et al.*, 2014; although see Voelckel & Baldwin, 2004; Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016). However, we found no evidence for such an interaction effect here for the performance of a third herbivore, *M. brassicae* (Table 3). This is in contrast to a study with powdery mildew and whiteflies on tomato plants that showed reverse effects of timing of arrival between the attackers when their order of arrival changed (Mouttet *et al.*, 2013). We did, however, find an interaction in feeding time and order of arrival for the inducing herbivores, but this did not directly translate to an effect on *M. brassicae* performance or preference (further discussed later). Apparently, in our case, the timing of herbivore inoculation shapes the plant phenotype such that it affects the performance of a subsequent feeder, but the order of herbivore species arrival did not matter. The mechanisms that underlie these effects remain to be elucidated.

Performance of inducers

In our study, we went beyond the focus of most studies on plant-mediated herbivore–herbivore interactions which study only the effects on two species, without including plant phenotypic consequences for subsequent feeders (Utsumi *et al.*, 2010; Stam *et al.*, 2014; Utsumi, 2015). Indeed, our data on performance of the two inducing herbivores also identify that they were affected by the presence of the other herbivore and that this was dependent on timing and order of arrival. Especially with a longer arrival time between inoculation of the aphid *B. brassicae* and the caterpillar *P. xylostella*, the aphids seemed to be affected more positively by interspecific feeding, while caterpillars were negatively affected by presence of the aphids (Figs 2 and 3). These results contrast with the general picture in the literature of a positive effect on caterpillar performance of aphid presence, and a negative effect of caterpillars on aphid performance (Stam *et al.*, 2014). These results reveal that induced plant responses are highly specific and could lead to asymmetric plant-mediated herbivore interactions that are not predicted by the feeding guild of the herbivore (Thaler *et al.*, 2002; Erb *et al.*, 2012; Soler *et al.*, 2012). Moreover, effects on performance of the inducing herbivores were not reflected in the performance of subsequent feeding by *M. brassicae*. This indicates that plant responses that mediate indirect interactions between multiple herbivores do not directly translate with the same magnitude and direction into responses of another herbivore (Utsumi *et al.*, 2010; Ohgushi, 2016). The response of a herbivore to an induced-plant phenotype depends on many aspects of plant–herbivore interactions, such as the species and feeding guild of inducing and responding herbivore (Bidart-Bouzat & Kliebenstein, 2011; Ali & Agrawal, 2014), the type of plant responses involved (Howe & Jander, 2008; Rodriguez-Saona *et al.*, 2010), and modifications of the plant response due to timing and order of arrival of multiple herbivores (Erb *et al.*, 2011; Karban, 2011; Stam *et al.*, 2014; Wang *et al.*, 2015). Therefore, going from two to multiple herbivores

in more natural situations of plant–herbivore communities cannot be interpreted by simple extrapolations (Stam *et al.*, 2014; Poelman, 2015).

Conclusion and future perspectives

In this study, we have shown that the order and timing of arrival of two herbivores on a plant change the plant's phenotype such that it affected a subsequent feeder in its food-plant choice and performance. The outcome of plant-mediated interactions among multiple herbivores in a community may thus be subject to variation in order and timing of arrival among herbivores. Major challenges in plant–insect interactions are to understand the mechanisms that shape these interaction networks and how these networks are reflected in evolutionary processes of plant–insect interactions (Utsumi *et al.*, 2010; Poelman, 2015; Ohgushi, 2016; Poelman & Kessler, 2016). To understand how plant responses to herbivory shape interaction networks, more knowledge is needed about the kinetics of plant responses to herbivory and how these physiological processes influence plant responses to multiple herbivore attack. Especially the time lag before onset of plant responses; changes in response strength during herbivore feeding; and the decay of plant response after feeding has stopped, are poorly understood (Gomez *et al.*, 2010; Karban, 2011; Underwood, 2012). Future studies should unravel the kinetics of plant responses to herbivores from different feeding guilds, which induce different types of plant response (Bidart-Bouzat & Kliebenstein, 2011; Erb *et al.*, 2011; Karban, 2011). Each of these aspects could determine how a plant responds to multiple herbivore attack by integrating physiological responses to multiple attackers.

Second, how these plant phenotypes, shaped by induction of multiple herbivores, determine food-plant preference of other community members is key to understanding how insect communities on individual plants are structured. It requires identification of whether herbivores select for plant phenotypes induced by multiple herbivores or whether their food-plant preference is determined by the presence of a specific key herbivore (Utsumi *et al.*, 2010; Ohgushi & Hambäck, 2015; Poelman & Kessler, 2016). The latter was identified for parasitic wasps in their search for hosts that were accompanied by multiple herbivore species on a single plant (de Rijk *et al.*, 2016). Plant physiological adaptations to multi-herbivore attack are expected to reflect the dynamics of plant-mediated interaction webs, and this requires the study of plant responses to more than two herbivores (Stam *et al.*, 2014; Wurst & Ohgushi, 2015; Poelman & Kessler, 2016).

Acknowledgements

We thank Léon Westerd, André Gidding, and Joop Woelke for rearing the insects and Unifarm for maintaining the greenhouses. The *B. oleracea* seeds were kindly provided by Rieta Gols. We are grateful for the valuable comments from Joop JA van Loon on an earlier version of this manuscript as well as to two anonymous reviewers for their constructive comments which greatly improved the manuscript. This study was supported by a

TOP GO grant (no. 854.10.010 to MD) from the Netherlands Organization for Scientific Research (NWO), funding from the European Research Council (ERC) under the European Union's Horizon 2020 research and innovation programme under grant agreement No 677139 to EHP, and a personal grant to LC, funded by the French Ministry of Research and Higher Education and delivered by the École Normale Supérieure de Lyon. The authors declare that they have no conflict of interest.

JMS, LC, MD and EHP designed the experiments; LC and JMS carried out the experiments; LC and JMS analysed the data; and JMS, LC, MD and EHP wrote the manuscript.

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Accepted 30 June 2017

Associate Editor: Takayuki Ohgushi