# Plasticity in Induced Resistance to Sequential Attack by Multiple Herbivores in Brassica Nigra

**Maite Fernández de Bobadilla** ( maria.fernandezdebobadilla@gmail.com )
Wageningen University & Research https://orcid.org/0000-0002-3542-4511
**Roel Van Wiechen**
Wageningen University and Research Wageningen Plant Research
**Gerrit Gort**
Wageningen University and Research Wageningen Plant Research
**Erik H. Poelman**
Wageningen UR: Wageningen University & Research

---

**Highlighted Student Paper - Original Research**

**Keywords:** sequential herbivory, multi-herbivore attack, canalisation, switching, defence plasticity, Plutella xylostella

**Posted Date:** February 4th, 2021

**DOI:** https://doi.org/10.21203/rs.3.rs-170192/v1



---

**Version of Record:** A version of this preprint was published at Oecologia on October 13th, 2021. See the published version at https://doi.org/10.1007/s00442-021-05043-1.

# Abstract

In nature, plants interact with multiple insect herbivores that may arrive simultaneously or sequentially. Because insect herbivores often have a negative effect on plant fitness, plants have evolved mechanisms to defend their tissues from being eaten. There is extensive knowledge on the regulation of induced plant responses to single or dual attack. However, we lack information on how plants defend against the attack of multiple herbivores that arrive sequentially. In this study, we investigated whether *Brassica nigra* plants are able to defend themselves against caterpillars of the late arriving herbivore *Plutella xylostella*, when plants had been previously exposed to sequential attack by four other herbivores. We manipulated the order of arrival and the history of attack by four herbivores to investigate which patterns in sequential herbivory determine resistance against the fifth attacker. We recorded that history of sequential herbivore attack differentially affected the capability of *Brassica nigra* plants to defend themselves against larvae of *P. xylostella.* A sequence of attack with four episodes of attack by *P. xylostella* induced resistance to larvae of *P. xylostella*. The number of times the plant was attacked by herbivores of the same feeding guild, the identity of the first attacker, the identity and the guild of the last attacker as well as the order of attackers within the sequence of multiple herbivores influenced plant resistance to subsequent herbivory. In conclusion, this study shows that history of sequential attack is an important factor determining plant resistance to herbivores.


# Introduction

In nature, plants interact with a species-rich community of insects (Giron et al., 2018). Some interactions with insects are beneficial for the plant, such as those with pollinators, whereas others are detrimental such as interactions with herbivores. Plants are exposed to the attack of multiple insect herbivores that feed from their tissues simultaneously or sequentially. Because insect herbivores often have a negative effect on plant fitness, plants have evolved defence mechanisms to prevent herbivores from eating their tissues (Erb, 2018). Some of those defence mechanisms are constitutive, and are always expressed independently of the presence of the attacker (War et al., 2012). However, maintaining defences is metabolically costly, and herbivores are very diverse in the way they consume the plant and in the defence traits they are sensitive to. Therefore, plants have evolved induced defences, that are only expressed upon attack and are more specific to the attacker compared to constitutive defence (Karban, 2011; Karban et al., 1997).

Studies on plants under single attack show specificity in the phytohormonal regulation of induced plant defence (Erb et al., 2012). The phytohormonal pathways that regulate induced plant responses cross-communicate with either positive, negative or neutral interactions between pathways (Pieterse et al., 2012; Soler et al., 2012; Thaler et al., 2012). The main phytohormones regulating plant responses to insects are jasmonic acid (JA) and salicylic acid (SA), but other phytohormones such as ethylene (ET) or abscisic acid (ABA) also play a role (Erb et al., 2012; Erb & Reymond, 2019). In general, plant responses to leaf-chewing insects are regulated via the JA pathway, whereas plant responses to phloem-feeding insects are regulated via the SA pathway (Erb & Reymond, 2019). JA and SA often have a negative crosstalk (Moreira et al., 2018). The crosstalk between phytohormones may compromise plant defences facing sequential attack by two insects of a different guild, where depending on the identity or guild of the first attacker the plant may become more resistant or more vulnerable to the attack of the second. Phytohormonal crosstalk between the SA and JA pathways often impairs plant responses to chewing herbivores when the plant has already directed its resistance response towards a phloem-feeding herbivore (Moreira et al., 2018; Soler et al., 2012). However, a recent study on dual attack testing 90 pairwise interactions between *Brassica nigra* plants and 10 herbivores shows that plants are not always limited to respond to chewers when they have been previously attacked by phloem feeders. Interestingly *B. nigra* plants adapt their induced response to the first attacker depending on the likelihood of subsequent attack determined by the prevalence of a second attacker in the field (Mertens, et al., 2021a,b). This study challenges the so-called JA-SA negative crosstalk paradigm, showing that to truly understand plant defence strategies to multiple herbivores many aspects still need to be explored.

One of the aspects of plant defence strategies that deserves more attention is how plants deal with the common situation of multi-herbivore attack. Only a few studies have explored plant defences to sequential herbivory beyond dual attack. These studies show that the order, the identity and the species richness of the attackers influences plant defence against a late arriving herbivore (Fernández de Bobadilla et al., 2021; Mathur et al., 2013; Stam et al., 2014, 2017, 2018, 2019). Nonetheless, in nature, herbivores often arrive sequentially, and studies including a realistic number of sequential attackers are lacking. This largely limits our understanding of how plastic plants adapt their defence phenotype to attackers that arrive in sequences. Several factors may determine the plant's ability to deal with sequential herbivore attack, such as the frequency of exposure to an attacker of a different feeding guild or the order of arrival of the attackers in the sequence such as the guild/identity of the first or last attacker in the sequence (Erb et al., 2011). Plants need to maximize their defence response to a current attacker in the context of an optimal response to the dynamic community of herbivores that may arrive later and also affect plant fitness (Mertens, et al., 2021; Poelman & Kessler, 2016).

The aim of this study was to investigate whether plants are able to defend themselves against a late arriving leaf chewing herbivore, when they had been previously exposed to sequential attack by four other herbivores. We subjected black mustard (*B. nigra*) plants to attack by 12 different sequences of herbivory or left the plants without herbivory and investigated plant defence against caterpillars of *Plutella xylostella*. We manipulated the number of times there were leaf-chewing or phloem-feeding insects in the four incidences of herbivore attack, as well as the order of the attackers and their species identity. We hypothesized that plants attacked more frequently by a leaf chewer would have a stronger JA induction and therefore would be better prepared to respond against a late arriving leaf chewer. Because the response to a first herbivore may most profoundly determine the capabilities of plants to deal with subsequent attack (Viswanathan et al., 2007), plants first attacked by a chewer may be better able to respond against a late arriving chewer compared to plants first attacked by a phloem feeding herbivore. Similarly, plants more recently attacked by a chewer may show a stronger induced defence towards chewers, compared with plants more recently attacked by a phloem feeder. Thus, we hypothesized that when the most recent attacker (i.e. the last in a sequence of four) was a chewer, plants would be more resistant to a late arriving chewer (fifth herbivore) than plants that had a phloem feeder as most recent attacker. Finally, we hypothesized that the order of the herbivores in the sequence would be an important factor in determining plant resistance against a late arriving herbivore.

## Materials And Methods

# Plants and insects

Two and a half weeks old black mustard plants (*B. nigra*, Brassicales: Brassicaceae) were used for the experiments. Seeds were obtained from a natural population in the vicinity of Wageningen (51° 57’ 32” N, 5° 40’ 23” E). The plants and the insects were grown and maintained in a greenhouse at 22 ± 2 °C, 60-70 % RH and 16:8 h L:D photo regime. Under natural conditions, individual *B. nigra* plants, a common annual in the Netherlands, are attacked by at least four up to twelve different herbivore species over their life-time (Mertens, et al., 2021b; Poelman et al., 2009). We used four herbivore species to simulate sequential attack: second instar larvae of the diamondback moth *Plutella xylostella* (*Px*) (Lepidoptera: Plutellidae) and first instar larvae the turnip sawfly, *Athalia rosae* (*Ar*) (Hymenoptera: Tenthredinidae) as leaf chewers and the cabbage aphid, *Brevicoryne brassicae* (*Bb*) and the green peach aphid, *Myzus persicae* (*Mp*) (both Hemiptera: Aphididae) as phloem feeders (Table 1). The degree of resistance induced by sequential attack by different sequences of attack by four herbivores was determined by assessing the performance of second instar larvae of the diamondback moth *P. xylostella* (Lepidoptera: Plutellidae) as fifth herbivore (that we call receiver). Larvae of this insect are specialists on brassicaceous plants and feed on foliar tissue, buds and flowers. *Plutella xylostella* typically arrives later in the vegetative growing season of *B. nigra* plants and often has to deal with plants previously damaged by other herbivores (Mertens et al., 2021b). *Brevicoryne brassicae* and *P. xylostella* were reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus). *Myzus persicae* and *A. rosae* were reared on radish (*Raphanus sativus*). All insects were obtained from the stock rearing of the Laboratory of Entomology, Wageningen University.

# Assessing plant resistance induced by a history of sequential attack by four herbivores

To test plant plasticity to multiple herbivore attack, we challenged *B. nigra* plants with several herbivore sequences and assessed whether responses to the herbivore sequences differentially affected induced plant resistance to subsequent herbivory by *P. xylostella* as reflected in the performance of caterpillars feeding on induced plants (Figure 1). We prepared a total of 18 plant replicates per herbivore combination divided in two blocks separated in time (nine plant replicates per herbivore combination in each block). We assessed induced plant responses to sequential attack by four herbivores, with a total of 12 treatments that differed in the order and identity of herbivore attack. For each of the four episodes of herbivory in a sequence, the total number used was three leaf chewers or six phloem feeders. The number of individuals per species initially introduced is representative for natural herbivore communities on *B. nigra* plants (Mertens et al., 2021b). We also prepared control plants that did not receive any inducing herbivore, but were otherwise treated in a similar way as plants receiving herbivores. Each herbivore episode in the sequence lasted for a period of five days, in which the herbivores were allowed to freely feed from the plant. After these five days all the inducers were removed with a fine brush, to exclude direct effects of inducing herbivores on the next herbivore in the sequence and on the receiving herbivore. By removing herbivores, we could focus on distinct events of herbivory, without aphid populations or chewing herbivores overexploiting plants due to the absence of control by their natural enemies. After plants had been exposed to their treatment of sequential attack by a sequence of four herbivore species and these herbivores had been removed, each plant was infested with 10 second instar larvae of *P. xylostella*, that acted as receiver to assess whether induced resistance to this herbivore was differentially affected by the history of herbivore attack (Figure 1). The mass of *P. xylostella* caterpillars was measured after five days of feeding on the induced plants as a proxy of plant resistance (19 $^{o}$C, 60-70 % RH and 16:8 h L:D photo regime). This was done by recapturing the *P. xylostella* larvae and weighing each individual on a Sartorius® - CP2P - Analytical Balance (accuracy 0.001 mg).

# Statistical analysis

## Performance data

To investigate whether the history of herbivory on *B. nigra* affected the growth of larvae of *P. xylostella*, we performed several analyses. For all tests we fitted a Mixed Linear Model (MLM), using as fixed effects the time blocks (with two levels) and appropriate part of the data grouped in order to answer the question of interest. Plants were included as random factors and residual error was also included. First, to assess whether the herbivore sequences influenced the plant´s capability to defend against larvae of *P. xylostella* feeding as fifth herbivore, we used fixed effects for all the treatments (with 13 levels: 12 herbivore sequences and a control). Second, to compare the treatments that received four times the same herbivores, we used fixed effects for the selected treatments (with five levels: control, and four sequences of either *P. xylostella, A. rosae, B. brassicae* or *M. persicae*). Third, to explore whether the number of times the plant was attacked by an insect of the same feeding guild affected its capability to defend against *P. xylostella*, we grouped our data based on the number of switches of chewers or of aphids in the four episodes of sequential herbivory, using this time fixed effects for number of sequences of chewers/aphids (with four levels: control, zero, two or four). For the rest of the analyses, we excluded the data from the treatments that received four times the same herbivore. We did so because we were interested in comparing the effect of herbivore sequences that contained an equal number of switches of attackers in the sequence. First, we used fixed effects for guild of the first or of the last attacker (with three levels: control, chewer or aphid). Then, we used fixed effects for species identity (with five levels: control, *P. xylostella, A. rosae, B. brassicae* and *M. persicae*). Finally, we tested for the effect of order of herbivores within the sequence, and compared treatments that received the same herbivores but in different order.

# Results

## History of sequential herbivore attack affects resistance against larvae of *P. xylostella*

The sequence of herbivore attack affected the capability of *B. nigra* plants to defend against larvae of *P. xylostella* ($F_{1,12}$=3.42, P<0.001, Figure 2). A sequence of attack by four episodes of leaf-chewing caterpillars of *P. xylostella* induced resistance to larvae of *P. xylostella* that were feeding on these plants as fifth herbivore ($t_{1,2}$=-2.17, P=0.032, Figure 2). No resistance was induced by a sequence of four episodes of attack by the other leaf chewer *A. rosae.* Performance of *P. xylostella* caterpillars feeding on plants induced by a history of four rounds of *A. rosae* did not differ from *P. xylostella* caterpillars feeding on plants that did not receive herbivory. Sequential attack by four rounds of aphid attack by either *M. persicae* or *B. brassicae* did not affect the performance of *P. xylostella* caterpillars as compared to performance on undamaged plants. However, specific sequences of herbivore attack affected the performance of *P. xylostella* caterpillars compared to performance on undamaged plants. Plants became more resistant to *P. xylostella* larvae after plants had been exposed to sequential attack by *M. persicae* – *M. persicae* – *A. rosae* – *A. rosae* ($t_{1,2}$=-4.42, P<0.001, Figure 2) and by *M. persicae* – *A. rosae* – *M. persicae* – *A. rosae* ($t_{1,2}$=-2.34, P=0.020, Figure 2).

## Importance of specific events in sequential attack for resistance to *P. xylostella* caterpillars

The differential effect of specific orders of sequential herbivore attack on performance of *P. xylostella* was determined by the number of switches between herbivore guilds, the identity of the first and last herbivore as well as the specific order of herbivore attack.

First, the number of times a plant was exposed to the same feeding guild of attacker as part of sequential herbivore attack, 0-, 2- or 4-times phloem feeders or leaf chewers affected plant resistance to larvae of *P. xylostella* ($F_{1,3}$=3, P=0.04). Plants that were attacked four times by chewers tended to be more resistant to larvae of *P. xylostella* than plants exposed to four times attack by phloem feeders ($t_{1,2}$=-1.67, P=0.097). Second, the feeding guild of the first attacker of the sequence did not affect plant resistance to larvae of *P. xylostella* (Figure 3a). However, the specific species identity of the first attacker of the sequence affected performance of *P. xylostella* (MLM: $F_{1,4}$=2.89, P=0.023, Figure 3b). Plants that had been attacked first by *M. persicae*, were more resistant to larvae of *P. xylostella* independent of the order and identity of the second, third and fourth herbivore in the sequence ($t_{1,2}$=-2.33, P=0.02, Figure 3b). Third, the feeding guild as well as the species identity of the last and thus fourth attacker of the sequence, affected plant resistance to *P. xylostella* larvae (MLM, Guild: $F_{1,3}$=-8.12, P<0.001; Identity: $F_{1,4}$=5.11, P<0.0001, Figure 3c, d). When the last attacker had been a chewer, plants were more resistant *P. xylostella* larvae (MLM, chewer $t_{1,2}$=-2.33, P=0.021) and these effects were particularly apparent for the leaf chewer *A. rosae* (MLM, $t_{1,4}$=-2.78, P=0.006, Figure 3c, d).

In summary, the history of sequential herbivore attack affected *B. nigra* resistance to larvae of *P. xylostella.* The number of times there was a chewer in the sequence, the species identity of the first attacker in the sequence and the guild and the identity of the last (i.e. fourth) attacker in the sequence affected the capability of plants to mount resistance to *P. xylostella* after previous exposure to multiple incidences of herbivore attack.

To further separate effects caused by number of switches between feeding guilds from effects by feeding guild, species identity and order of arrival, we analysed these effects within subsets of treatments that were equal in the number of herbivore switches. *Brassica nigra* plants attacked by sequences containing two times chewers, and two times phloem feeders, were more vulnerable to *P. xylostella* larvae when the first attacker was a chewer, compared with plants that were first attacked by a phloem feeder ($F_{1,2}$= 10.85, P=0.001). After first attack by a phloem feeder, the order of subsequent attackers influenced plant resistance to *P. xylostella*, as larvae grew more on plants attacked by the sequence aphid – chewer – aphid – chewer (*B. brassicae* – *P. xylostella* – *B. brassicae* – *P. xylostella* as well as *M. persicae* – *A. rosae* - *M. persicae* – *A. rosae*) than on plants attacked by the sequence aphid – aphid – chewer – chewer (*B. brassicae* – *B. brassicae* – *P. xylostella* – *P. xylostella* as well as *M. persicae* – *M. persicae* – *A. rosae* – *A. rosae*) (MLM, $F_{1,2}$=5.06, P=0.03; Figure 4a). When the first attacker was the phloem feeder *B. brassicae,* plants were equally vulnerable to larvae of *P. xylostella,* irrespectively of the order of the subsequent attackers ($F_{1,2}$=1.34 P= 0.25, Figure 4c). In contrast, when the first attacker was *M. persicae* the order of the attackers mattered and larvae of *P. xylostella* grew more on plants where the identity of the herbivore switched every time (larvae grew more on plants that had been exposed to *M. persicae – A. rosae – M. persicae – A. rosae* than on plants that had been exposed to *M. persicae – M. persicae – A. rosae – A. rosae*) ($F_{1,2}$=4.08 P=0.04, Figure 4d). In contrast to the effect of herbivore order when the first attacker was an aphid, the order of herbivore arrival after the first herbivore was a leaf chewer did not affect plant resistance to *P. xylostella* larvae ($F_{1,2}$=0.75 P=0.39, Figure 4b). These effects were also similar for the two leaf chewers *P. xylostella* or *A. rosae* (*P. xylostella* first: $F_{1,2}$=0.05 P=0.83; *A. rosae* first: $F_{1,2}$=2.08 P=0.15, Figure 4e, f).

In summary, *B. nigra* plants attacked by sequences containing two chewers, and two phloem feeders, were more vulnerable to *P. xylostella* larvae when the first attacker was a chewer, compared with plants that were first attacked by a phloem feeder. Additionally, when plants were first attacked by an aphid, the order of subsequent attackers in the sequence influenced plant resistance. Plants that were attacked by the sequence aphid – chewer – aphid – chewer were more vulnerable to *P. xylostella* larvae than plants attacked by the sequence aphid – aphid – chewer – chewer.

## Discussion

The objective of this study was to investigate plant resistance against a leaf chewing herbivore, after plants had been previously exposed to sequential attack by four other herbivores. We found that sequence of herbivore attack differentially affected *B. nigra* plant resistance to *P. xylostella* larvae. Four events of attack by *P. xylostella* induced resistance to larvae of *P. xylostella* compared to control plants. The number of times the plant was attacked by herbivores of the same feeding guild, the identity of the first attacker, the identity and the guild of the last attacker as well as the order of attackers within the sequence of multi herbivory influenced plant resistance to subsequent herbivory. The guild of the first attacker of the sequence did not affect plant resistance to *P. xylostella*. However, when plants had been first attacked by *M. persicae* they were more resistant to larvae of *P. xylostella,* regardless of the order of other herbivores attacking the plant. In contrast, the guild and identity of the last attacker influenced plant resistance to *P. xylostella* larvae as they grew less on plants where the last attacker of the sequence had been a chewer, especially when it was *A. rosae.* Our study shows that the sequence of herbivore attack is an important factor determining plant resistance to herbivores.

In line with the induced defence hypothesis, *B. nigra* plants that had been attacked four times by caterpillars of *P. xylostella* were more resistant to caterpillars of *P. xylostella* feeding as fifth herbivore. However, when the aphid *B. brassicae* was introduced in the sequence of four attackers, the induced resistance to larvae of *P. xylostella* disappeared. The compromised induced resistance when there are other attackers than *P. xylostella* in the sequence of four attackers, suggests that *B. nigra* plants loose potential to deal with a specific herbivore attack when switching defence machinery towards other attackers in the sequence. Furthermore, four exposures to attack by the other leaf chewer (*A. rosae*) did not make the plant more resistant to *P. xylostella* caterpillars*.* The absence of induced resistance by the other chewer indicates that the induced response found on plants attacked four times by *P. xylostella* is not just a general defence mechanism in response to chewers, but that there is specificity in induced defence within feeding guilds. Similar specificity in induced resistance was found for *Solanum dulcamara*. Plants that had been damaged by the leaf-chewing beetle *Psylliodes affinis* were more resistant to *P. affinis*, while feeding by the leaf chewer *Plagiometriona clavata* did not induce resistance against *P. affinis* (Viswanathan et al., 2005). Our work identifies that specificity of induction by herbivore identity may be maintained under multi-herbivore attack.

Plants that were sequentially attacked four times by aphids defended equally well against larvae of *P. xylostella* compared with plants that had not been exposed to herbivory. This indicates that when the plant suffered four rounds of aphid attack, there was no aphid-induced susceptibility to a chewer. Several studies of plant responses to dual attack, have reported aphid-induced susceptibility to chewers, often supporting their findings based on the JA/SA negative crosstalk paradigm (Davidson-Lowe et al., 2019; Koornneef & Pieterse, 2008; Li et al., 2014; Rodriguez-Saona et al., 2005; Soler et al., 2012). The absence of aphid-induced susceptibility to caterpillars or even presence of aphid-induced resistance to caterpillar attack may be caused by aphids depleting nutrients in the plant or by shifts in secondary metabolites (Jakobs et al., 2019). It is becoming clear that not only the guild of previous attackers is important in explaining plant resistance but many other factors such as the density of attackers influence the outcome of plant mediated interactions between herbivores (Kroes et al., 2015; Pineda et al., 2017). For resistance of *B. nigra* to sequential herbivore attack the prevalence of the second herbivore in the field is a more important driver of plant induced responses to the first attacker than the identity of the first herbivore itself (Mertens et al., 2021b).

Our work highlights that also the number of times the plant was attacked by an insect of the same feeding guild within the sequence of four attackers, influenced the plant's capability to respond to attack by *P. xylostella*. Caterpillars grew bigger when feeding on plants that had been exposed to four events of aphid infestation compared with those feeding on plants that had been exposed to four events of chewer attack or compared with plants that had been exposed to two rounds of herbivory by aphids plus two by chewers. This suggests that when the plant suffers attack by herbivores that arrive in sequences, being more times attacked by one type of insect, makes the plant more ready to defend against an insect of a similar type. Moreover, plants attacked by sequences containing two times chewers, and two times phloem feeders, were more vulnerable to *P. xylostella* larvae when the first attacker was a chewer, compared with plants that were first attacked by a phloem feeder. This suggests that the feeding guild of the first attacker may influence the plant's capability to defend against subsequent attackers. In line with our results, in maize the order of herbivore arrival was important in determining plant resistance to sequential attack. *Spodoptera frugiperda* attack induced resistance against larvae of *Diabrotica virgifera virgifera*, but only when *S. frugiperda* attacked the plant first (Erb et al., 2011). In our study, the species identity of the first attacker partly influenced plant resistance, as plants that had been attacked first by *M. persicae* were more resistant to *P. xylostella* larvae. Moreover, when the last attacker of the sequence was a chewer, especially when it was *A. rosae,* the plant was more resistant to *P. xylostella* larvae. Common garden experiments monitoring herbivore communities on *B. nigra* show that *M. persicae* is one of the first attackers colonising *B. nigra* plants, and that *A. rosae* and *P. xylostella* arrive later in the growing season of the plant (Mertens et al., 2021b). The fact that we found herbivore-induced resistance in response to exposure to herbivore sequences that are more commonly found in the field, suggests that *B. nigra* plants are adapted to the natural order of herbivore arrival (Mertens et al., 2021b).

Several studies on plant responses against single herbivore attack show canalization of plant responses, where plants attacked by a herbivore cannot fully defend after sequential attack (Soler et al., 2012; Viswanathan et al., 2007). Canalization may not be the optimal defence strategy in a scenario of multiple attack by herbivores that arrive sequentially. If the plant completely directs its defence machinery towards the first herbivore, and cannot switch response to the upcoming attackers, the plant may be undefended against later arriving herbivores. Our work does not show evidence for canalization of plant defences in *B. nigra*, as there is no induced susceptibility to *P. xylostella* larvae by sequences of four herbivores, compared with undamaged plants. Consequently, when facing herbivory by multiple insects that arrive in sequences, the ability of *B. nigra* plants to defend against a late arriving herbivore, is not hampered. Additionally, our data suggest that *B. nigra* plants do not fully switch their resistance phenotype to a new attacker, i.e. plants that had been attacked four times by *P. xylostella* were more resistant to larvae of *P. xylostella* but when there was a switch of attackers in between, the induced resistance was reduced. Furthermore, *P. xylostella* larvae grew better on plants attacked by the sequence aphid – chewer – aphid – chewer than on plants attacked by the sequence aphid – aphid – chewer – chewer. The reduced resistance of plants that had been exposed to more switches of attackers, further supports that *B. nigra* plants are limited in showing a full defence response when the attacker changes several times.

To conclude, we show that history of sequential attack is an important factor determining plant resistance to its community of attackers. The relative importance of overlapping herbivore populations, densities, timing of their arrival and plant ontogeny, in addition to patterns in incidence we tested here, should be evaluated in future studies. In depth studies on the physiological changes after exposure to each newly arriving herbivore should shed light on how plants regulate plasticity to multi-herbivore attack. Additionally, it is crucial that further studies explore plant adaptation to multi-herbivore attack under field conditions. This could be done by studying the herbivore communities forming on plants previously induced by different sequences of herbivory (Stam et al., 2018) assessing the importance of the first attacker, the last attacker and the order of attackers within the sequence and assessing the consequences for other community members and on plant fitness.

## Declarations

# Acknowledgements

The authors want to thank the Unifarm team for growing the plants, especially Sean Geurts and André Maassen. We thank Marcel Dicke for constructive comments on an earlier version of the manuscript.

**Funding**: This research was funded by the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (grant agreement n° 677139) to EHP.

**Conflicts of interest/Competing interests**: The authors declare no conflict of interests

**Ethics approval (include appropriate approvals or waivers)**: All applicable institutional and/or national guidelines for the care and use of animals were followed.
**Consent to Participate**: not applicable

**Consent for publication**: not applicable

**Availability of data and material**: Data will be available upon reasonable request to the corresponding author.

**Authors’ contributions**: MFB and EHP conceived and designed the experiments. MFB and RvW performed the experiments. MFB, and GG analysed the data. MFB and EHP wrote the manuscript.

## References

1. Davidson-Lowe, E., Szendrei, Z., & Ali, J. G. (2019). Asymmetric effects of a leaf-chewing herbivore on aphid population growth. *Ecological Entomology, 44*(1), 81–92. https://doi.org/10.1111/een.12681

2. Erb, M. (2018). Plant Defenses against Herbivory: Closing the Fitness Gap. *Trends in Plant Science*, *23*(3), 187–194. https://doi.org/10.1016/j.tplants.2017.11.005
3. Erb, M., Meldau, S., & Howe, G. a. (2012). Role of phytohormones in insect-specific plant reactions. *Trends in Plant Science*, *17*(5), 250–259. https://doi.org/10.1016/j.tplants.2012.01.003
4. Erb, M., & Reymond, P. (2019). Molecular Interactions between Plants and Insect Herbivores. *Annual Review of Plant Biology*, *70*, 527–557. https://doi.org/10.1146/annurev-arplant-050718-095910
5. Erb, M., Robert, C. A. M., Hibbard, B. E., & Turlings, T. C. J. (2011). Sequence of arrival determines plant-mediated interactions between herbivores. *Journal of Ecology*, *99*(1), 7–15. https://doi.org/10.1111/j.1365-2745.2010.01757.x
6. Fernández de Bobadilla, M., Bourne, M. E., Bloem, J., Kalisvaart, S. N., Gort, G., Dicke, M., & Poelman, E. H. (2021). Insect species richness affects plant responses to multi-herbivore attack. *New Phytologist*. https://doi.org/https://doi.org/10.1111/nph.17228
7. Jakobs, R., Schweiger, R., & Müller, C. (2019). Aphid infestation leads to plant part-specific changes in phloem sap chemistry, which may indicate niche construction. *New Phytologist*, *221*(1), 503–514. https://doi.org/10.1111/nph.15335
8. Karban, R. (2011). The ecology and evolution of induced resistance against herbivores. *Functional Ecology*, *25*(2), 339–347. https://doi.org/10.1111/j.1365-2435.2010.01789.x
9. Karban, R., Agrawal, A. A., & Mangel, M. (1997). The benefits of induced defenses against herbivores. *Ecology*, *78*(5), 1351–1355. https://doi.org/10.1890/0012-9658(1997)078[1351:TBOIDA]2.0.CO;2
10. Koornneef, A., & Pieterse, C. M. J. (2008). Cross talk in defense signaling. *Plant Physiology, 146*(3), 839–844. https://doi.org/10.1104/pp.107.112029
11. Kroes, A., Van Loon, J. J. A., & Dicke, M. (2015). Density-dependent interference of aphids with caterpillar-induced defenses in arabidopsis: Involvement of phytohormones and transcription factors. *Plant and Cell Physiology, 56*(1), 98–106. https://doi.org/10.1093/pcp/pcu150
12. Li, Y., Dicke, M., Harvey, J. A., & Gols, R. (2014). Intra-specific variation in wild *Brassica oleracea* for aphid-induced plant responses and consequences for caterpillar-parasitoid interactions. *Oecologia, 174*(3), 853–862.
13. Mathur, V., Tytgat, T. O. G., de Graaf, R. M., Kalia, V., Sankara Reddy, A., Vet, L. E. M., & van Dam, N. M. (2013). Dealing with double trouble: Consequences of single and double herbivory in *Brassica juncea*. *Chemoecology*, *23*(2), 71–82. https://doi.org/10.1007/s00049-012-0120-z
14. Mertens, D., Boege, K., Kessler, A., Thaler, J. S., Whiteman, N. K., & Poelman, E. H. (2021). Predictability of Biotic Stress Structures Plant Defence Evolution. *Trends in Ecology & Evolution*. https://doi.org/https://doi.org/10.1016/j.tree.2020.12.009
15. Mertens, D., Fernández de Bobadilla, M., Rusman, Q., Bloem, J., Douma, J. C., & Poelman, E. H. (2021). Plant defence to sequential attack is adapted to prevalent herbivores. *Submitted*.
16. Moreira, X., Abdala-Roberts, L., & Castagneyrol, B. (2018). Interactions between plant defence signalling pathways: Evidence from bioassays with insect herbivores and plant pathogens. *Journal of Ecology*, *106*(6), 2353–2364. https://doi.org/10.1111/1365-2745.12987

17. Pieterse, C. M. J., Van der Does, D., Zamioudis, C., Leon-Reyes, A., & Van Wees, S. C. M. (2012). Hormonal Modulation of Plant Immunity. *Annual Review of Cell and Developmental Biology*, *28*(1), 489–521. https://doi.org/10.1146/annurev-cellbio-092910-154055
18. Pineda, A., Soler, R., Pastor, V., Li, Y., & Dicke, M. (2017). Plant-mediated species networks: the modulating role of herbivore density. *Ecological Entomology, 42*(4), 449–457. https://doi.org/10.1111/een.12404
19. Poelman, E. H., & Kessler, A. (2016). Keystone Herbivores and the Evolution of Plant Defenses. *Trends in Plant Science21*(6), 477-485. https://doi.org/10.1016/j.tplants.2016.01.007
20. Poelman, E. H., Van Dam, N. M., Van Loon, J. J. A., Vet, L. E. M., & Dicke, M. (2009). Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores. *Ecology*, *90*(7), 1863–1877. https://doi.org/10.1890/08-0977.1
21. Rodriguez-Saona, C., Chalmers, J. A., Raj, S., & Thaler, J. S. (2005). Induced plant responses to multiple damagers: Differential effects on an herbivore and its parasitoid. *Oecologia, 143*(4), 566–577. https://doi.org/10.1007/s00442-005-0006-7
22. Soler, R., Badenes-Pérez, F. R., Broekgaarden, C., Zheng, S. J., David, A., Boland, W., & Dicke, M. (2012). Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: From insect performance to gene transcription. *Functional Ecology*, *26*(1), 156–166. https://doi.org/10.1111/j.1365-2435.2011.01902.x
23. Stam, J. M., ChrÉtien, L., Dicke, M., & Poelman, E. H. (2017). Response of *Brassica oleracea* to temporal variation in attack by two herbivores affects preference and performance of a third herbivore. *Ecological Entomology*, *42*(6), 803–815. https://doi.org/10.1111/een.12455
24. Stam, J. M., Dicke, M., & Poelman, E. H. (2018). Order of herbivore arrival on wild cabbage populations influences subsequent arthropod community development. *Oikos*, *127*(10), 1482–1493. https://doi.org/10.1111/oik.05265
25. Stam, J. M., Kos, M., Dicke, M., & Poelman, E. H. (2019). Cross-seasonal legacy effects of arthropod community on plant fitness in perennial plants. *Journal of Ecology*, *107*(5), 2451–2463. https://doi.org/10.1111/1365-2745.13231
26. Stam, J. M., Kroes, A., Li, Y., Gols, R., van Loon, J. J. a, Poelman, E. H., & Dicke, M. (2014). Plant interactions with multiple insect herbivores: from community to genes. *Annual Review of Plant Biology*, *65*, 689–713. https://doi.org/10.1146/annurev-arplant-050213-035937
27. Thaler, J. S., Humphrey, P. T., & Whiteman, N. K. (2012). Evolution of jasmonate and salicylate signal crosstalk. *Trends in Plant Science*, *17*(5), 260–270. https://doi.org/10.1016/j.tplants.2012.02.010
28. Viswanathan, D. V., Lifchits, O. A., & Thaler, J. S. (2007). Consequences of sequential attack for resistance to herbivores when plants have specific induced responses. *Oikos*, *116*(8), 1389–1399. https://doi.org/10.1111/j.2007.0030-1299.15882.x
29. Viswanathan, D. V., Narwani, A. J. T., & Thaler, J. S. (2005). Specificity in induced plant responses shapes patterns of herbivore occurrence on *Solanum dulcamara*. *Ecology, 86*(4), 886–896. https://doi.org/10.1890/04-0313

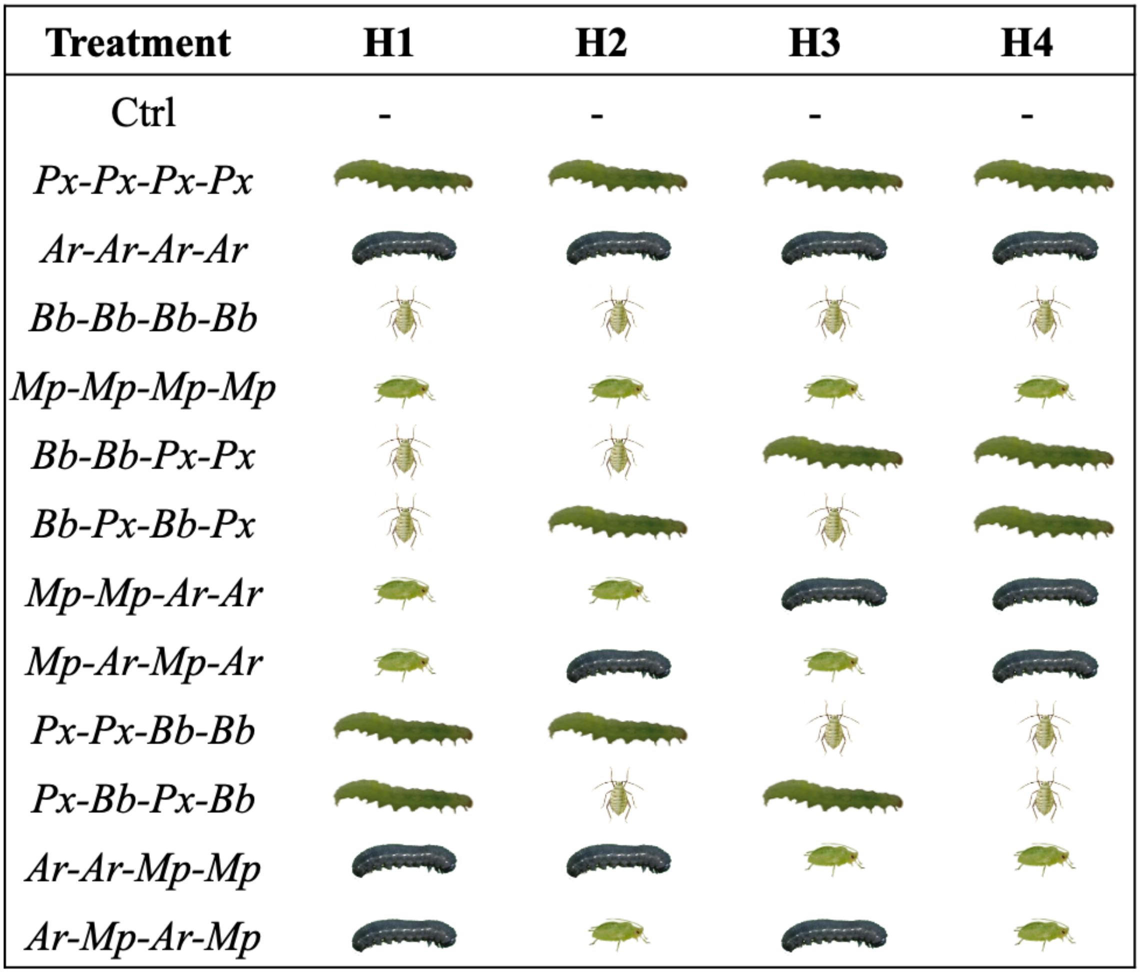
30. War, A. R., Paulraj, M. G., Ahmad, T., Buhroo, A. A., Hussain, B., Ignacimuthu, S., & Sharma, H. C. (2012). Mechanisms of plant defense against insect herbivores. *Plant Signaling & Behavior*, *7*(10), 1306–1320. https://doi.org/10.4161/psb.21663

# Tables

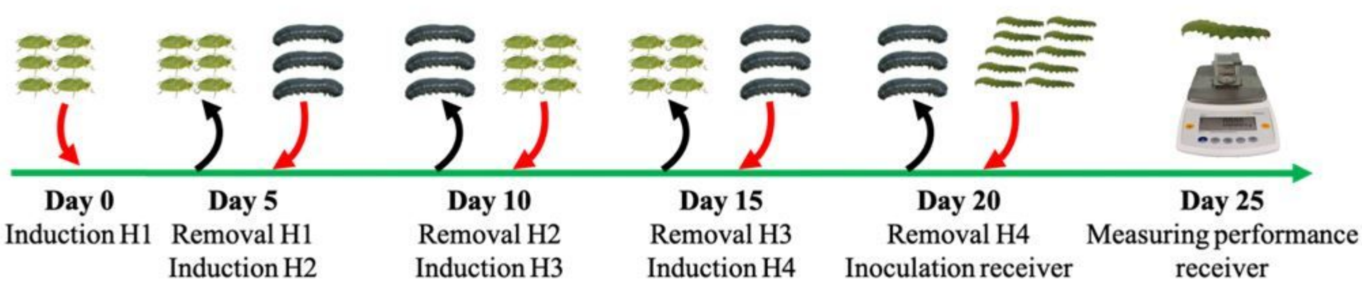
**Table 1.** Insects used for the experiment, in brackets abbreviations used throughout the document.

| Species (Abbreviation) | Picture | Order | Feeding guild |
|---|---|---|---|
| *Athalia rosae (Ar)* |  | Hymenoptera | Leaf chewer |
| *Plutella xylostella (Px)* |  | Lepidoptera | Leaf chewer |
| *Brevicoryne brassicae (Bb)* |  | Hemiptera | Phloem feeder |
| *Myzus persicae (Mp)* |  | Hemiptera | Phloem feeder |

**Table 2.** List of herbivore sequences (H) applied to *Brassica nigra* plants to assess plant resistance to *Plutella xylostella*. Leaf chewers*: Px= P. xylostella, Ar= Athalia rosae.* Aphids*: Bb= Brevicoryne brassicae, Mp= Myzus persicae.*




# Figures




### Figure 1

Experimental timeline depicting the herbivore sequences. On Day 0 each Brassica nigra plant was induced with the first set of herbivores (H1) which consisted of either three chewers or six aphids. After five days all the herbivores were removed from the plant using a brush, and the second set was introduced (H2). This was done for four events of an herbivore sequence. After the herbivores of the last round of herbivory (H4) had been removed from the plant, 10 larvae of Plutella xylostella were introduced on each plant and they were allowed to feed from the induced plants for five days. Each P. xylostella larva was recaptured and weighed as a measure as of plant resistance.

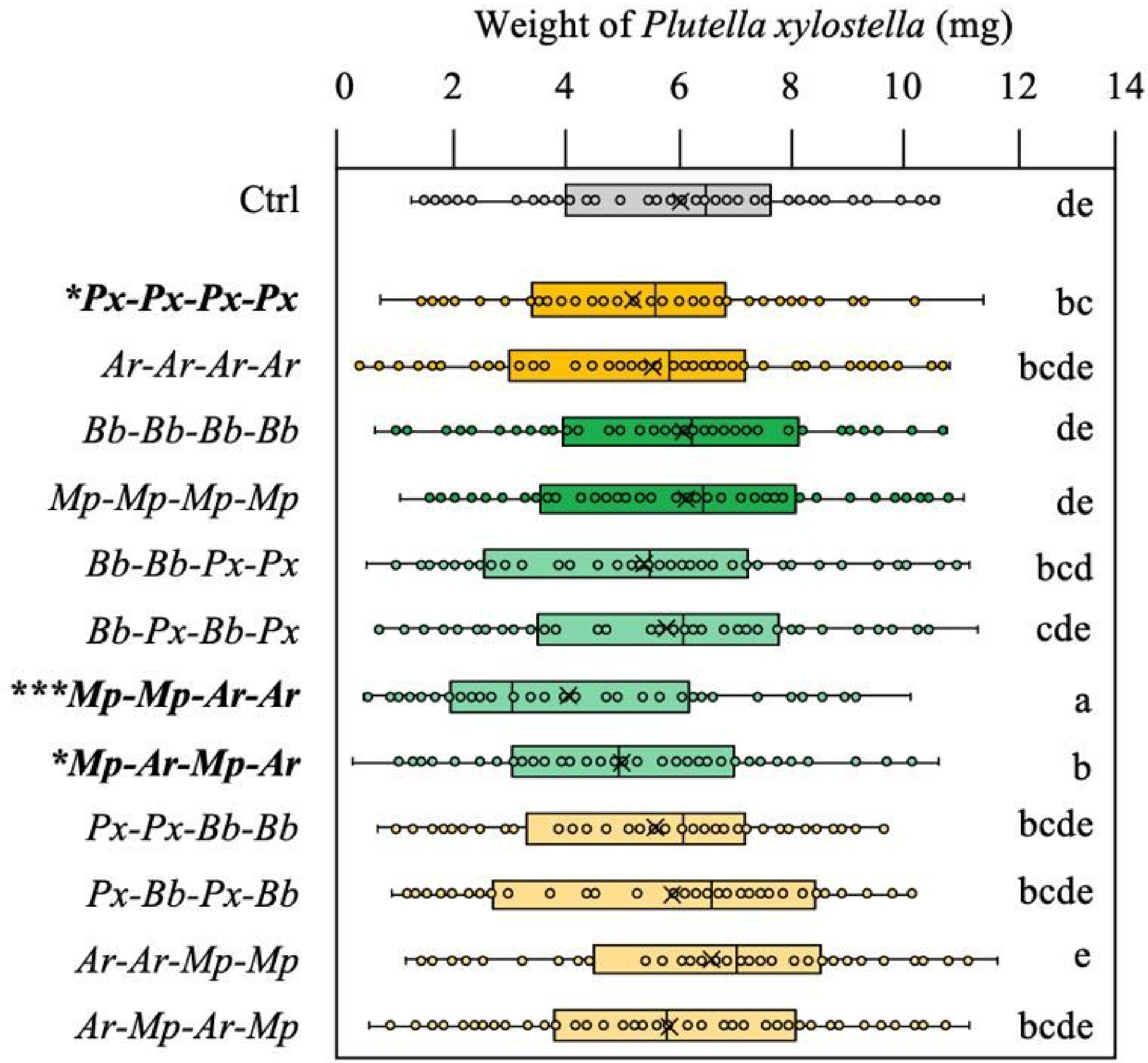
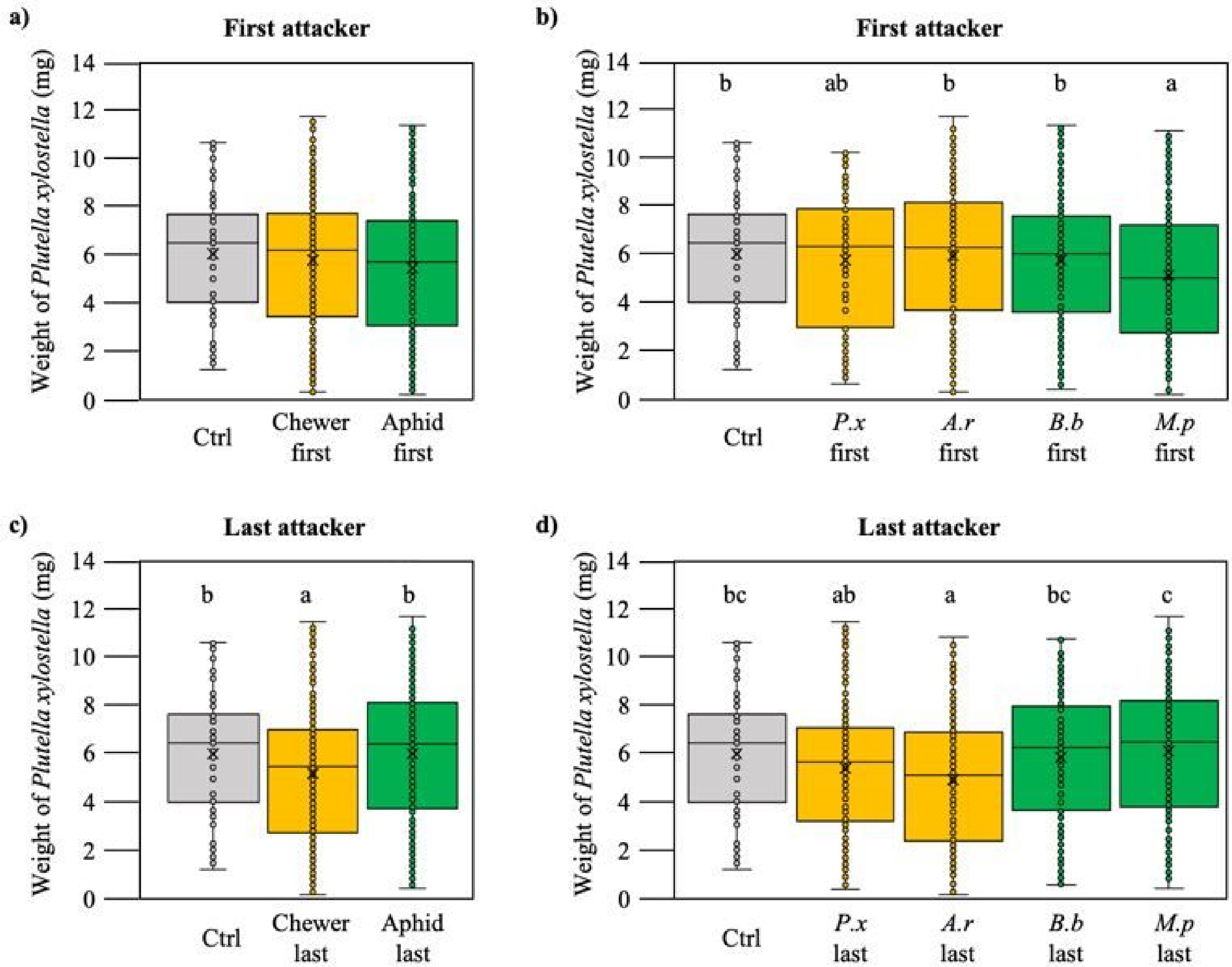



**Figure 2**

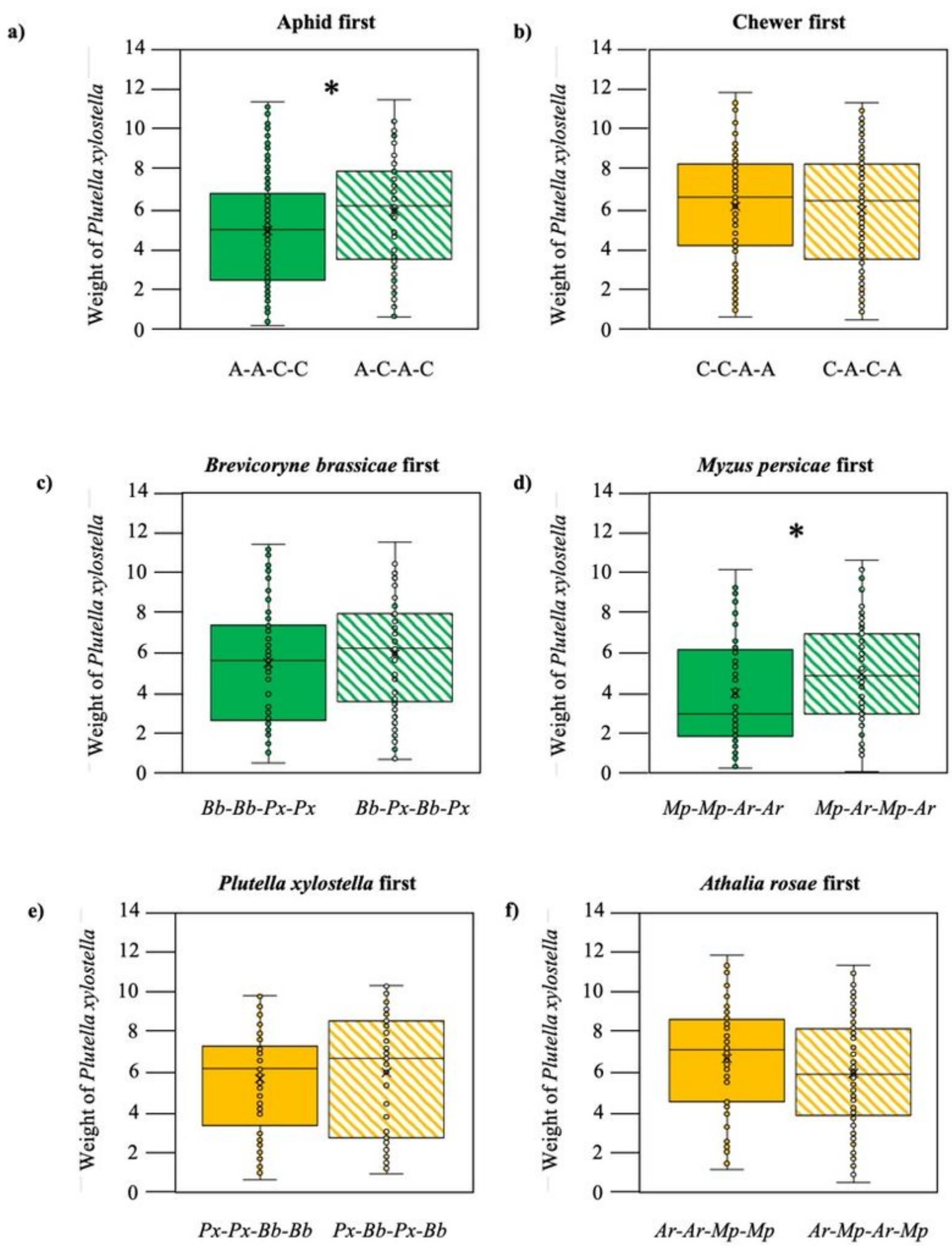
Weight (mg) of Plutella xylostella larvae after feeding for five days from Brassica nigra plants previously attacked by sequences of four herbivore events (N= 18). Dark yellow and dark green boxplots indicate herbivore sequences that received four times a chewer or a phloem feeder, respectively. Light yellow and light green boxplots indicate herbivore sequences of two chewers plus two phloem feeders where the first attacker was a chewer or a phloem feeder, respectively. Herbivore sequences that affected P. xylostella growth (compared to control, untreated plants) are marked in bold and with asterisks with significance levels *P<0,05; ***P<0,001. Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. Letters above the boxplots show significant differences (MLM, posthoc Tukey).

Leaf chewers: Plutella xylostella (Px) and Athalia rosae (Ar). Phloem-feeding aphids Brevicoryne brassicae (Bb) and Myzus persicae (Mp).




## Figure 3

Effect of first or last attacker on resistance to Plutella xylostella. Weight (mg) of P. xylostella larvae after feeding as fifth herbivore from Brassica nigra plants that were previously attacked by four sets of herbivores where: a) the first attacker was either a chewer, an aphid or untreated plants (Ctrl). b) The first attacker was Plutella xylostella (Px), Athalia rosae (Ar), Brevicoryne brassicae (Bb), Myzus persicae (Mp), or untreated plants (Ctrl). c) The last attacker was either a chewer, an aphid or untreated plants (Ctrl). d) The last attacker was Plutella xylostella (Px), Athalia rosae (Ar), Brevicoryne brassicae (Bb), Myzus persicae (Mp), or untreated plants (Ctrl). Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. Letters above the boxplots show significant differences (MLM, posthoc Tukey).




## Figure 4

Weight (mg) of Plutella xylostella larvae after feeding for five days from Brassica nigra plants attacked by a sequence of herbivory with the guild or the species identity of first attacker constant but changing the order of the subsequent attackers of the sequence. First attacker: a) an aphid (A), b) a chewer (C), c) Brevicoryne brassicae (Bb) d) Myzus persicae, (Mp) e) Plutella xylostella (Px), d) Athalia rosae (Ar).

Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. Asterisks show comparisons with significance differences *P<0,05.