

Plant defence strategies to multi-herbivore attack

Maite Fernández de Bobadilla



Propositions

1. Herbivore species richness in multi-herbivore attack on plants influences plant resistance to a subsequent attacker, the plant-associated insect community and plant fitness.
(this thesis)
2. Plants tailor their defence strategies to the anticipated arrival of the most prevalent herbivores.
(this thesis)
3. Transferring knowledge to society rather than to the rest of the scientific community is the first priority for scientists.
4. Current measures of scientific success are not representative for scientific quality.
5. Discriminatory behaviours originate from either ignorance or the desire to maintain privileges.
6. Collaboration between people is the way to achieve the Sustainable Development Goals.

Propositions belonging to the thesis entitled 'Plant defence strategies to multi-herbivore attack'

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Thesis

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CHAPTER 1
General Introduction

INTRODUCTION

Plants live in a complex environment where they interact with a community of diverse organisms ranging from microbes to mammals. Insects are very important members of that community of organisms because of the number of interactions and the large effect they have on plant fitness (Cozzolino *et al.*, 2015; Lankau & Strauss, 2008; Soper Gorden & Adler, 2018). Some of the interactions with insects are detrimental for the plant such as interactions with herbivores (Grass *et al.*, 2018), whereas others are beneficial such as interactions with natural enemies of the herbivores and with pollinators (Gervasi & Schiestl, 2017; Ollerton *et al.*, 2011). Herbivores often have a direct negative effect on plant fitness, because they consume plant tissues and reduce the photosynthetic capacity of the plant (Nabity *et al.*, 2009). In contrast, natural enemies of the herbivores, such as parasitoids are hypothesized to have a positive effect on the plant, because they control the populations of the herbivores that consume plant tissues (Romero & Koricheva, 2011). Additionally, interactions with pollinators have an enormous impact on plant fitness, because the majority of flowering plants depend on pollinators for reproduction (Ollerton *et al.*, 2011). In order to maximise their fitness, plants need to prevent herbivores from eating their tissues while maintaining interactions with beneficial organisms (Grass *et al.*, 2018; Herrera *et al.*, 2002). In nature, plants are attacked by multiple insect herbivores that induce changes in plant phenotypic traits and affect plant interactions with other organisms such as other herbivores, natural enemies of the herbivores and pollinators (Moreira *et al.*, 2019). However, how plants defend themselves against the attack of multiple insects while maintaining the attraction of parasitoids and pollinators in order to maximise fitness, is poorly understood (Dicke *et al.*, 2009).

PLANT DEFENCE AGAINST HERBIVORES

To protect their tissues from being eaten, plants are equipped with constitutive defence traits that are always present independent of the presence of the attacker. If a herbivore attacks the plant, another layer of defence comes into play and plants change their phenotype by expression of an induced



defence response, that is tailor made to the current attacker (Karban, 2011). This induced response will directly target the attacker, and aims to reduce the damage the attacker inflicts to the plant, known as direct defence. The induced response will not only directly target the herbivore, but may also attract natural enemies of the herbivore, such as predators and parasitoids, known as indirect defence (Bustos-Segura *et al.*, 2020; Karban *et al.*, 1997). Induced plant resistance to herbivory is modulated by the identity (Agrawal *et al.*, 2014; Van Zandt & Agrawal, 2004), guild (Bidart-Bouzat & Kliebenstein, 2011; Davidson-Lowe *et al.*, 2019), and density of the attacker (Kroes *et al.*, 2016; Kroes *et al.*, 2015; Pineda *et al.*, 2017; Ramirez & Eubanks, 2016), among others. Only recently, studies including more than two attackers have unravelled that the order of attackers also influences a plant's ability to defend against attackers (Stam *et al.*, 2017, 2018, 2019; Viswanathan *et al.*, 2007). Nonetheless, we are far from understanding how plants defend themselves against the attack of more than two herbivores or in other words multi-herbivore attack.

MOLECULAR REGULATION OF PLANTS TO DEAL WITH HERBIVORE ATTACK

Plants can sense virtually every external stimulus surrounding them (Kliebenstein, 2014). Plant interactions with their biotic environment are regulated in an integrated system, where information travels at the molecular level (gene, transcript, metabolite) and at the morphological level (cells, tissues and the whole plant) (Atkinson & Urwin, 2012). Irrespective of whether interactions with organisms are beneficial, detrimental or neutral for the plant, plants can detect all of them. Detection is mediated by plant receptors that recognise specific molecules present in the organisms and activate a regulatory response. The molecules that trigger a response in the plant have been named associated molecular patterns (AMPs), and depending on the organism (or organism part) they are called PAMPs, MAMPs, HAMPs, DAMPs or EAMPs when they come from a pathogen, a microorganism, a herbivore, damage or an egg, respectively (Acevedo *et al.*, 2015; Gouhier-Darimont *et al.*, 2019; Jones & Dangl, 2006; Kliebenstein, 2014). When feeding from the plant, HAMPs and DAMPs come into contact with the inner part of the cell.



HAMPs include molecules present in the insect oral secretion that elicit a defence response whereas DAMPs are cell fragments mixed with intracellular components. Together the HAMPs and DAMPs resulting from the leaf-chewing process by the herbivore, characterize the signature from chewing insects (Acevedo *et al.*, 2015; Erb & Reymond, 2019). The different AMPs bind to specific pattern recognition receptors (PRRs) in the plant, which will start a defence response. Several HAMPs have been identified in insect oral secretions (Schmelz *et al.*, 2006; Truitt *et al.*, 2004). However, so far only PRRs for DAMPs have been described (Erb & Reymond, 2019). Early signalling events triggered by herbivory include 1) depolarization of the plasma transmembrane potential, 2) rise in cytosolic Ca^{2+} ; 3) production of reactive oxygen species (ROS) and 4) mitogen activated protein kinase (MAPK) activity. The early signalling activated upon herbivore attack is connected to broad transcriptional rearranging and a defence response regulated by hormonal signalling networks (Maffei *et al.*, 2007).

Despite the specificity in herbivore recognition (especially well-supported in plant-pathogen interactions) the signalling cascade that regulates phenotypic changes induced by herbivore attack seems more general. The Jasmonic Acid (JA) signalling is the core pathway regulating plant defence responses, especially against chewing insects.

JA biosynthesis occurs via the lipoxygenase pathway which is activated upon mechanical damage and herbivory. Upon damage, acyl-lipidic hydrolases release α -linolenic acid from the plastid membrane, which is the substrate for the enzyme lipoxygenase (LOX) which activates the expression of *LOX* genes (Bell *et al.*, 1995).

A key regulator of the first steps in JA biosynthesis is the enzyme lipoxygenase (LOX) which activates expression of JA-biosynthetic genes, such as ***LOX2*** (Bell *et al.*, 1995; Sarde *et al.*, 2018). 12-oxophytodienoic acid (OPDA) is generated and transported to the peroxisome, where it undergoes three β -oxidation steps and forms (+)-iso-JA that is transported to the cytosol and



conjugates with isoleucine. JA-isoleucine (JA-Ile) is the bioactive form of JA that is transported to the nucleus where it binds to nuclear receptors, formed by CORONATINE-INSENSITIVE1 (COI1), JASMONATE-ZIM DOMAIN (JAZ), and an inositol-polyphosphate cofactor. The binding of JA-Ile to JAZ triggers JAZ degradation by the proteasome, which in turn releases the repression of MYC transcription factors, resulting in expression of JA-responsive genes, such as *VSP2*, which are involved in expression of defence genes against a wide diversity of herbivores (Erb & Reymond, 2019). Salicylic acid (SA), ethylene (ET) and abscisic acid (ABA) are well-known stress-related phytohormones that are also induced upon herbivory and are modulators of the defence response. Moreover, growth hormones, such as gibberellins (GAs), auxin and cytokinins (CKs) are involved in antiherbivore responses. GAs regulate growth via proteasome-mediated degradation of DELLA repressors, which modulate the JA pathway. Activation of the GA pathway leads to DELLA degradation and inhibition of JA responses (Erb & Reymond, 2019).

In general, plant-induced responses to attack by insects of the chewing feeding guild are regulated via the JA-phytohormonal pathway whereas plant responses to attack by phloem feeders are regulated via the SA-pathway (Erb *et al.*, 2012; Jaouannet *et al.*, 2014; Züst & Agrawal, 2016). Deficiency in SA makes tomato plants more susceptible to *Myzus persicae* whereas SA-deficient *Arabidopsis* plants are not affected in resistance (Erb & Reymond, 2019) and mutants in the JA biosynthetic pathway generally show reduced resistance against chewers (Ye *et al.*, 2012). The different signalling pathways interact and can stimulate or inhibit each other. The best described example is the negative crosstalk between SA and JA. Several plant species that have been attacked by phloem-feeding insects cannot defend against a chewer as well as undamaged plants do (Moreira *et al.*, 2018; Pieterse *et al.*, 2012; Soler *et al.*, 2012). In addition, ET/JA and ABA/ET antagonism has also been reported, and this further modulates plant defence against herbivores (Verhage *et al.*, 2011). Additionally, transcription factors play a key role in regulating defences both up- and downstream of the phytohormone signalling. Downstream of phytohormonal signal-transduction, induced plant



secondary metabolites can regulate plant defence. For example, benzoxazinoids in maize induce callose deposition (Kliebenstein, 2014).

Herbivore-induced plant phenotypic changes start at the damaged site (Chandran *et al.*, 2010). At the cell level, cell autonomous responses to the organism will be first transported to neighbouring cells, to the whole leaf, and to the whole plant to form a systemic response (i.e. in distal plant tissues). The communication in plants is not limited to a systemic signal within one individual. Plants interact and communicate with their neighbouring plants via volatiles and via the roots, exchanging information useful to survive, such as the presence of danger (Babikova *et al.*, 2013; Farmer & Ryan, 1990). Importantly, there is also interkingdom communication between plants and micro- and macro-organisms. Striking examples of interkingdom communication are plants detecting and discriminating fungal volatiles from different soil-borne fungi, and modulating root growth direction (Moisan *et al.*, 2021) or parasitoids differentiating volatiles from plants attacked by their host from undamaged plants or plants attacked by a different herbivore (De Rijk *et al.*, 2016a,b). Herbivory induces dramatic changes in a plant phenotype, and because plants communicate and interact with a whole range of organisms, it is not surprising that herbivory alters the dynamics of the plant-associated community (Pashalidou *et al.*, 2015; Soper Gorden & Adler, 2018; Stam *et al.*, 2014).

HERBIVORE INDUCED CHANGES ON THE INSECT COMMUNITY

The induced response against one herbivore does not only affect the current attacker, but may also affect the attraction and the performance of other insects that colonise the plant later including other herbivores, natural enemies of the herbivores and pollinators (Cozzolino *et al.*, 2015; Ohgushi, 2008; Rusman *et al.*, 2018; Van Zandt & Agrawal, 2004). As all these community members may have strong effects on plant fitness, this change in interactions can have dramatic effects for the plant (Cozzolino *et al.*, 2015; Glaum & Kessler, 2017; Kessler & Chautá, 2020; Soper Gorden & Adler, 2018). Consequently, plant



responses to herbivory can alter community dynamics and plant trait selection (Lankau & Strauss, 2008; Strauss, 2014; Strauss & Irwin, 2004).

Parasitoids are generally hymenopteran or dipteran species that predate on other insects, and therefore are classified as natural enemies of the herbivores. The foraging of parasitoids is highly affected by herbivore-induced changes in a plant phenotype. Upon attack by insect herbivores, plants release herbivore-induced plant volatiles that attract parasitoids, acting as a “cry for help” (Dicke & Baldwin, 2010). Parasitoids are able to discriminate between plants that have been attacked or not, and even on the type and density of attackers (De Boer *et al.*, 2008; De Rijk *et al.*, 2013, 2016a-c). In nature, parasitoids need to find their host in a complex environment, where some plants are attacked by hosts, others by non-host herbivores and yet others may even be attacked by several attackers (Aartsma *et al.*, 2017, 2019). We know that parasitoids are able to discriminate between plants under single or dual attack (Blubaugh *et al.*, 2018; Ponzio *et al.*, 2016; Rodriguez-Saona *et al.*, 2003, 2005), however, we lack studies investigating whether parasitoid foraging is affected by multiple attack. This knowledge will help us better understand parasitoid foraging under more natural conditions.

Importantly, plant defence against herbivores may trade off with pollinator attraction. Herbivore-induced changes in the volatile blend of the plant can also affect attraction of pollinators. Additionally, herbivory induces changes in important cues for pollinator recruitment such as flower colour/shape/odour, pollen and nectar quality (Lucas-Barbosa, 2016; Lucas-Barbosa *et al.*, 2016; Nicholls & Hempel de Ibarra, 2017; Rusman *et al.*, 2018, 2019 a,b). Field studies show that the community of pollinators of *B. nigra* plants changes when the plants are under single herbivore attack early in the season. Interestingly, plant-mediated interactions between herbivores and pollinators are different depending on the species of herbivore attacking the plant (Rusman *et al.*, 2018). These studies show that plant responses to single herbivore attack can affect pollinator attraction, but we lack information whether the response of the pollinators is affected by multiple herbivore attack.



GENERAL OBJECTIVE AND RESEARCH QUESTIONS OF THIS THESIS

The aim of my PhD project, was to explore how *Brassica nigra* plants deal with multiple attack by its most common herbivores. In my thesis I explored four main questions (Fig. 1):

- 1) Can plants anticipate arrival of the most prevalent herbivores and tailor their induced defence strategies accordingly? (**chapter 3**)
- 2) Does species richness of previous attackers affect a plant's capability to respond to a subsequent attacker of the same or of a different feeding guild? (**chapter 4**)
- 3) Does species richness of previous attackers affect plant interactions with herbivores, parasitoids, pollinators and do these effects result into plant fitness consequences in the field? (**chapter 5**)
- 4) Are plants able to defend against a late arriving herbivore after a history of sequential attack by four other herbivores? (**chapter 6**)

STUDY SYSTEM

The black mustard, *Brassica nigra*

As a focal plant I used the wild annual plant *Brassica nigra* (Brassicaceae family). This pretty plant contains yellow flowers, is a fast growing species, highly branched, and an obligatory outcrossing species. Its leaves and flowers contain glucosinolates, secondary metabolites characteristic for the Brassicaceae involved in defence against herbivores and attraction of pollinators. The extensive information on the ecology and mechanistic machinery of *B. nigra* available makes *B. nigra* an excellent focal plant to address the main questions of my PhD. First, the herbivore community of *B. nigra* plants is well characterised (Mertens *et al.*, 2020 thesis chapters 3,6; Mertens *et al.*, 2021; Poelman *et al.*, 2009). Additionally, some studies addressed herbivore-induced responses to single attack in *B. nigra* (Van Dam *et al.*, 2005; Van Dam & Raaijmakers, 2006). There is even modelling exercises made to assess the plant growth defence trade-off under conditions of herbivory and competition for light (de Vries *et al.*, 2017, 2018, 2019). Second, the pollinator community of *B. nigra* plants, as well as the effects of single attack by



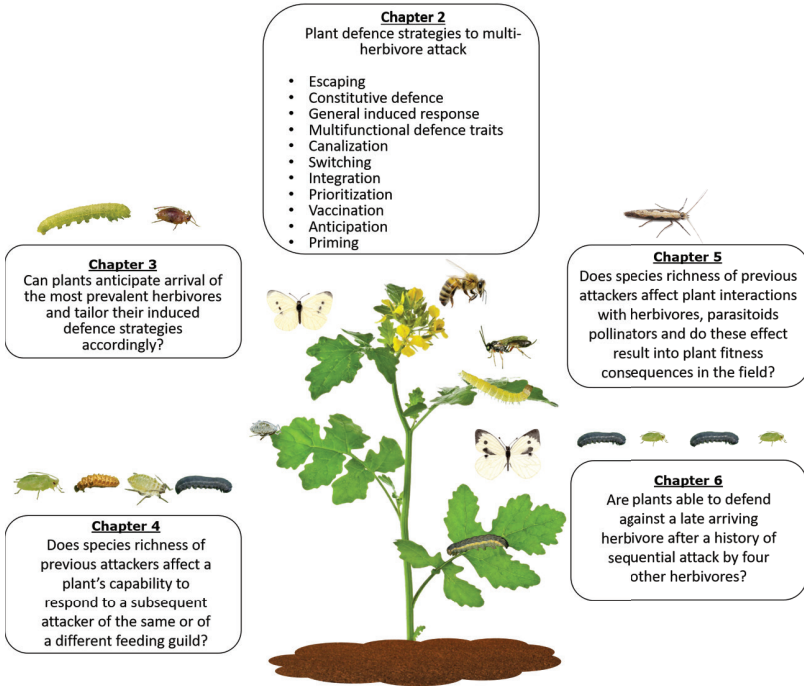


Figure 1. Overview of the main questions investigated in each chapter of the thesis.



different herbivores and the effects of dual attack on the pollinator community have also been explored (Chrétien *et al.*, 2021; Rusman *et al.*, 2018, 2020). Third, there is information on how community complexity shapes selection on traits in *B. nigra* (Lankau & Strauss, 2008). Fourth, other studies have addressed mechanistic and molecular interactions of *B. nigra* with several stressors. These studies include transcriptomic responses to single attack by insects of different feeding guilds (Broekgaarden *et al.*, 2011), metabolic and phytohormonal profile after dual attack (Chrétien *et al.*, 2018; Papazian *et al.*, 2019; Ponzio *et al.*, 2017), and transcriptomic responses to dual attack (Bonnet *et al.*, 2017). Even recently, the genome of *B. nigra* has been sequenced, allowing for a more in-depth study of the molecular regulation of this plant's responses to interactions with members of the associated community (Perumal *et al.*, 2020). Thus, the ecological and molecular knowledge basis of *B. nigra* plants, allowed me to design ecologically relevant experiments related to the plant's responses to multi-herbivore attack.

Insect herbivores used in the experiments

To study plant adaptations to the attack by multiple insect herbivores, I challenged my plants with 11 insect herbivores (Table 1). I applied herbivores with a chewing feeding guild and with a phloem feeding guild. All the herbivores I used in my experiments are commonly found in the field attacking *B. nigra* plants (Mertens *et al.*, 2021) and therefore may be important selection agents on this plant species (Table 1).












Chewers

As chewing insects, I used seven insect species, including larvae of Lepidoptera, Coleoptera and Hymenoptera. Among them there were two generalist species and five species specialized on brassicaceous plants.

The silver Y *Autographa gamma*, and the cabbage moth *Mamestra brassicae* (both Lepidoptera: Noctuidae) are generalist insects, their larvae are polyphagous and attack Brassicaceae plants but also Leguminosae, Solanaceae and many other plants.



Table 1. Insect herbivores used for the experiments ordered according to their prevalence in the field (% total plants where the insect was observed). Records of the most common insect herbivores observed on *Brassica nigra* plants during three years of field experiments: 2011, 2012 and 2013. Each year 180 plants were monitored for insect presence. Insect presence was assessed by weekly counts, surveying herbivore community development on individual plants from seedling until seed maturation. Adapted from (Mertens *et al.*, 2021).

Picture	Species	Order	Diet breadth	Feeding guild	Total number of individuals	Average number per plant	Median per plant	Prevalence (% of plants)
	<i>Plutella xylostella</i>	Lepidoptera	Specialist	Chewer	5155	11	9	98.4
	<i>Myzus persicae</i>	Hemiptera	Generalist	Phloem feeder	10364	22	19	98.2
	<i>Brevicoryne brassicae</i>	Hemiptera	Specialist	Phloem feeder	867877	2243	351	79.3
	<i>Pieris rapae</i>	Lepidoptera	Specialist	Chewer	1437	4	3	74.8
	<i>Mamestra brassicae</i>	Lepidoptera	Generalist	Chewer	391	2	1	40.2
	<i>Lipaphis erysimi</i>	Hemiptera	Specialist	Phloem feeder	327	3	2	22.1
	<i>Phaedon cochleariae</i>	Coleoptera	Specialist	Chewer	1580	15	6	21.1
	<i>Athalia rosae</i>	Hymenoptera	Specialist	Chewer	78	1	1	11.7
	<i>Myzus persicae</i> sub. <i>nicotianae</i>	Hemiptera	Generalist	Phloem feeder	147	3	1	11.1
	<i>Autographa gamma</i>	Hemiptera	Generalist	Chewer	75	3	2	5.9
	<i>Pieris brassicae</i>	Lepidoptera	Specialist	Chewer	251	23	1	2.3



The turnip sawfly *Athalia rosae*, (Hymenoptera: Tenthredinidae) is a specialist herbivore of brassicaceous plants. Female wasps lay eggs on the leaf edge and upon hatching larvae feed voraciously until they consume all the leaf tissue and move to another leaf. Larvae and adults of the mustard leaf beetle *Phaedon cochleariae* (Coleoptera: Chrysomelidae) are herbivores specialised on brassicaceous plants. Adults emerge in large numbers, and due to the high mobility and voracious feeding they can devastate fields of Brassicaceae plants. When larvae have acquired enough body mass, they burry themselves in the soil for pupation. Butterflies of the large white *Pieris brassicae* (Lepidoptera: Pieridae) lay eggs in clutches on the leaf surface of Brassicaceae plants (Fatouros *et al.*, 2015). Upon hatching, the larvae feed gregariously from that same leaf and when they reach the second instar they move to the buds and flowers. Butterflies of the small cabbage white *Pieris rapae* (Lepidoptera: Pieridae) lay single eggs on brassicaceous plants and larvae feed from leaves. The diamondback moth *Plutella xylostella* (Plutellidae) is a specialist insect of plants of the Brassicaceae family with a short generation time and high fecundity. Larvae of *P. xylostella* cause big economic losses worldwide. Female moths of *P. xylostella* are attracted to glucosinolate breakdown products and lay many eggs on brassicaceous plants. Upon hatching, larvae burrow a mine inside the leaf, and after a few days they leave the mine and start chewing the whole leaf.

Phloem-feeding insects

As phloem-feeding insects, I used four aphid species, all belonging to the Hemiptera (Aphididae). The cabbage aphid *Brevicoryne brassicae* is a specialist of brassicaceous plants; they can reach big colonies of thousands of individuals on leaves, stems, buds and flowers. The mustard turnip aphid, *Lipaphis erysimi*, is also a specialist insect that can be found mostly on lower stems, but also on leaves and flowers of brassicaceous plants. The green peach aphid, *Myzus persicae* is a very generalist phloem feeder, that can be found on more than 400 plant species, including brassicaceous plants. The red tobacco aphid, *Myzus persicae* sub. *nicotianae* is an important pest on tobacco cultivars, although it feeds from many other plant species, including *B. nigra*.



OUTLINE OF THIS THESIS

In **chapter 2**, I propose a framework for plant defence to multi-herbivore attack by defining the repertoire of plastic defence strategies that may allow plants to optimize defence against multiple herbivore attack. These defence strategies could be seen as risk management tools to deal with a multitude of stressors.

In **Chapter 3**, I investigated whether the level of induced resistance to sequential herbivory in *B. nigra* corresponds with the likelihood of the order and nature of sequential insect attack in the field. I linked observations of the order of arrival of insect herbivores during three years of season-long insect community assessment on 488 plants, to a full-factorial assessment of induced resistance in pairwise plant-mediated interactions between ten herbivore species. I specifically tested whether induced resistance of the second herbivore can be predicted by traits (feeding guild and diet breadth) of the inducing herbivore or the secondary receiving herbivore species, as well as the commonality of those species and their pairwise interactions in the field (Mertens *et al.*, 2021). Moreover, to characterise the specificity of induced plant response, I studied the expression of the JA-biosynthesis marker gene (*LOX2*) and of the SA-responsive gene (*PR1*) after plants were attacked by the ten herbivores.

In **chapter 4**, I investigated whether species richness of previous attackers affects a plant's capability to respond to a subsequent attacker of the same or of a different feeding guild. I challenged *B. nigra* plants with 51 treatments representing attack by an increasing species richness (1, 2 or 4 species) of either phloem feeders, leaf chewers, or a mix of both feeding guilds when keeping total density of attackers constant. I selected eight of the most prevalent herbivore species equally distributed across the leaf chewer and phloem feeder guilds and studied how plant responses to these attackers affect resistance to subsequent attack by caterpillars of *P. xylostella*. Additionally, to characterise the specificity of the induced plant responses to the 51 herbivore combinations I studied the expression of the JA-biosynthesis marker gene (*LOX2*), of the JA-responsive gene (*VSP2*), of the SA-biosynthesis gene (*ICS*) and of the SA-responsive gene (*PR1*) 48 and 96h after herbivore attack.



In **chapter 5**, I studied how herbivore species richness and its trait composition affect plant interactions with its insect community and whether these interactions affect plant fitness. I conducted a manipulative field study where I subjected *B. nigra* plants to attack by an insect herbivore community with increasing species richness from one and/or two feeding guilds (i.e. phloem feeders, leaf chewers or a mix of both). I specifically studied how the composition of the introduced herbivore community affected 1) plant colonisation by the herbivore *P. xylostella*, 2) top-down control of this herbivore's population by parasitoids, 3) visitation of flowers by pollinators, and 4) how these interactions collectively affect plant fitness in terms of seed production. I examined the relations between the different community members and effects of varying levels of herbivore richness and feeding guild by applying structural equation modelling (SEM). This allowed to partition the direct and indirect effects of different community members on plant fitness, and how early-season herbivore attack alters these relationships.

In **chapter 6**, I investigated whether *B. nigra* plants are able to defend themselves against caterpillars of the late-arriving herbivore *P. xylostella*, when plants had been previously exposed to sequential attack by four other herbivores. I 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. I specifically studied the effect of: 1) the identity and the guild of the first attacker; 2) the identity and the guild of the last attacker; 3) the order of arrival of herbivores in a sequence 4) the number of times a plant is attacked by herbivores of the same feeding guild.

Finally, in **chapter 7**, I summarize and discuss the main contributions of my PhD to the field of insect-plant interactions. Furthermore, I give a perspective for future research.

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CHAPTER 2 Plant defence strategies to multi-herbivore attack

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HIGHLIGHTS

- Virtually all plants are under attack by a species rich community of antagonists. By recognizing the individual attacker, plants may effectively tailor their defence response. However, the response to one attacker should be balanced with the costs that this response may entail towards defence against other attackers.
- Reprogramming of plant physiology to counteract herbivory is typically studied under single or at most dual attack. Often these studies identify antagonistic interactions that limit plants in their ability to resist a second attacker. The emerging physiological concepts may mismatch with the adaptive nature of plant responses in community context.
- Identifying plant plasticity to deal with their full community of attackers is key in understanding plant defence strategies and requires unified terminology to illustrate the repertoire of plant plasticity matching ecological patterns of attack.

ABSTRACT

Plants may effectively tailor defences by recognizing their attackers and reprogramming their physiology. Although most plants are under attack by a large diversity of herbivores, surprisingly little is known about the physiological capabilities of plants to deal with multi-herbivore attack. Studies on dual herbivore attack identified that defence to one attacker may cause energetic and physiological constraints to deal with a second attacker. How these constraints shape plant plasticity in defence to multi-herbivore attack and what defence strategies plants may deploy to their full community of attackers is a major knowledge gap in plant science. Here we provide a framework for plant defence to multi-herbivore attack by defining the repertoire of plastic defence strategies that may allow plants to optimize defence against a multitude of stressors.



PLANTS ARE UNDER SELECTION TO DEFEND AGAINST MULTI-HERBIVORE ATTACK

Plants interact with a large diversity of organisms in antagonistic or mutualistic relationships. For many plant species, insect herbivores are some of the most prominent and diverse antagonists that often have a direct or indirect negative impact on **plant fitness** (see Glossary) (Atkinson & Urwin, 2012). To maximise their fitness, plants use tolerance and resistance strategies (Schuman & Baldwin, 2016; Erb, 2018). An important asset of the defence repertoire in plants is that they can recognize the specific attacker (Acevedo *et al.*, 2015; Erb and Reymond, 2019; Züst & Agrawal, 2016) and mount **induced responses** to tailor resistance to the type of attacker. Such induced responses are likely a cost saving strategy (Karban, 2020). Typically, responses of plants to herbivores are studied by isolating responses to individual stressors. These studies yielded extensive knowledge on plant perception, recognition and regulation of responses to insect attack (Box 1). Over the past decades, studies on dual herbivore attack uncovered interactions between gene regulatory networks involved in response to individual stressors, resulting in distinct responses to multiple attackers (Voelckel & Baldwin, 2004; Vos *et al.*, 2015; Zarate *et al.*, 2007; Zhang *et al.*, 2009). Importantly, these studies also identified that antagonistic cross communication between these networks may impair plants in maximising resistance to individual stressors, may allow herbivores to exploit the cross communication to suppress plant defence and may result in susceptibility to herbivory (Caarls *et al.*, 2015; Li *et al.*, 2015; Li *et al.*, 2018; Proietti *et al.*, 2018; Thaler *et al.*, 2012). While these results represent a step forward to understand defence responses in a herbivore community context, they do not yet capture the full dynamics occurring in nature, where plants are simultaneously or sequentially attacked by a multitude of organisms (Stam *et al.*, 2014). In theory, plants should optimise their defence strategies to deal with communities of attackers (Mertens *et al.*, 2021; Orrock *et al.*, 2015; Poelman & Kessler, 2016). However, clear examples and a framework for plant defence optimization towards herbivore communities from a physiological perspective are currently missing. Here, we provide a framework to understand plant adaptive responses to multi-herbivore attack.



GLOSSARY

Allocation costs: reductions in growth or reproduction as a direct consequence of diversion of limiting resources to defence processes

Defence costs: cost associated to the expression of defence traits or to the cost of expressing the traits in the absence of plant enemies: allocation, ecological or physiological costs, decreases in fitness resulting from interactions with other species.

Dynamic attacker community: multiple attackers can arrive simultaneously, sequentially, and on high and low numbers.

Ecological costs: costs of plant's defence associated with changes in interactions with community members.

Induced response: defence trait that is activated upon herbivore attack and recognition.

Metabolic costs: energy and resources required for the production and maintenance of defences.

Plant defence trait: plant trait that increases the fitness of plants upon herbivore attack

Plant fitness: contribution of an individual plant to the gene pool of the next generation

Tolerance: ability of a plant to reduce fitness losses of herbivory without preventing the attack.

PLASTICITY TO MATCH COMMUNITY CONTEXT

From an adaptive point of view, plants should not only balance their investment in defence with maintaining competitiveness for light and nutrients, they should tailor their responses to maximize fitness upon herbivore attack (Ballaré *et al.*, 2019; de Vries *et al.*, 2017; Züst & Agrawal, 2017). As plants are attacked by communities of herbivores, this implies that plant responses should be balancing the costs and benefits of their responses in a community context (Mertens *et al.*, 2021a,b; Poelman & Kessler, 2016). Thus, defence



strategies may be advanced risk management strategies that are analogous to chess games to deal with a multitude of stressors (Mertens *et al.*, 2021b; Orrock *et al.*, 2015).

Specific or generalized responses to herbivory

When considering that plants will be attacked by a multitude of herbivores, it should be questioned to what extent plants recognise and respond to specific attackers or that the responses target a broader range of multiple attackers (Erb *et al.*, 2012). There is substantial evidence that plants recognize herbivore feeding guilds by their type of damage (piercing-sucking or leaf chewing), specific herbivores by associated elicitors and that plants differentially respond to individual herbivores (Schuman & Baldwin, 2016; Acevedo *et al.*, 2015; Erb & Reymond, 2019) (Box 1). However, there is also considerable overlap in core transcription profiles after herbivore attack and little evidence that the expressed phenotype specifically targets the current attacker (Mertens *et al.*, 2021a). Limited specificity may be particularly adaptive under high and predictable herbivore pressure. When herbivore pressure is high, plants express their full defences *constitutively* to reduce herbivory at high maintenance costs that may have a negative impact on plant growth and fitness (Züst & Agrawal, 2017) (Box 2). To reduce maintenance costs under high and predictable herbivore pressure, plants may use a *general induced response* that targets all potential attackers at once. Several studies have shown that individual herbivores induce similar suits of defence compounds, where activity of individual compounds may only effectively target a single herbivore species (Adio *et al.*, 2011; Gonzales-Vigil *et al.*, 2011). This suggests that either these plant species were not capable of recognizing the specific attacker and thus mount a broad spectrum of defences, or that individual plants are predictably attacked by most of the herbivore species in the pool of potential attackers (Mertens *et al.*, 2021b). The costs associated with mounting a broad palette of defence compounds may be reduced when compounds are highly *multifunctional* and are involved in direct and indirect defence as well as in primary metabolism (Neilson *et al.*, 2013; Quintana-Rodríguez *et al.*, 2018).



BOX 1: Plant perception, recognition and response to herbivores

To deal with multiple attackers, that may all require different **defensive traits**, plants have evolved mechanisms to recognize the specific attacker they are facing (Acevedo *et al.*, 2015; Schuman & Baldwin, 2016). Plants perceive herbivory via binding and recognition of specific molecules. These molecules may originate from disrupted cell walls that provide general information on plant damage (damage-associated molecular patterns, DAMPS) or provide more specific information on herbivore identity by herbivore-associated molecular patterns (HAMPS) in herbivore saliva for example. Perception and recognition of specific attackers results in mobilisation of resistance through phytohormonal signalling and broad transcriptional reprogramming (Acevedo *et al.*, 2015; Erb & Reymond, 2019; Schuman & Baldwin, 2016). Each attacker elicits a unique signature of responses involving a large number of phytohormones. In general, responses to leaf-chewing herbivores such as caterpillars are predominately mediated by Jasmonic Acid (JA), Abscisic Acid (ABA) and Ethylene (ET). Phloem-feeding herbivores such as aphids or biotrophic pathogens typically induce responses that are mediated by Salicylic Acid (SA) induction (Erb & Reymond, 2019; Erb *et al.*, 2012).

Crosstalk between signal-transduction pathways allows plants to fine-tune responses to multiple attackers (Thaler *et al.*, 2012; Vos *et al.*, 2015). The JA/ET pathway is activated by JASMONATE ZIM-DOMAIN (JAZ) proteins that regulate JA activity and thereby transcriptional activators. The JA pathway has two branches, the MYC2 branch activates wound-induced responses through transcription of *LOX*. The ERF branch is connected to JA and ET signalling and activates transcription of *PDF1.2* that is primarily involved in responses to necrotrophic pathogens (Hickman *et al.*, 2017). Signalling through one branch has antagonistic effects on the other branch. SA accumulates after attack by sap-sucking herbivores and regulates *NPR1* and its downstream WRKY and TGA transcription factors. These activate defence gene expression such as those that encode for pathogenesis related proteins (*PR1*). Crosstalk between the signal-transduction pathways is strongly regulated by the *NPR1* proteins and WRKY transcription factors that link the JA and SA pathways (Koorneef and Pieterse, 2008). Signal transduction in the SA pathway has antagonistic effects on JA regulation (Caarls *et al.*, 2015). This may result in aphid-induced susceptibility to caterpillar attack (Ali & Agrawal, 2014; Soler *et al.*, 2012).



When it is highly unpredictable when, by which herbivore species and in which order attack will take place, adaptive plant defence strategies may include specific responses to an attacker (Karban, 2020). Theoretically, a more specific response maximizes resistance to the current attacker and reduces investment costs to only the specific defences needed (Karban, 2020). However, a specific response to one herbivore may impair plants in maximising resistance or even result in susceptibility to other herbivores thus compromising fitness. Plant regulation of responses to attack by different herbivore feeding guilds is involved in antagonistic cross communication by which aphids may reduce plant resistance to caterpillars (Ali & Agrawal, 2014; Fernández de Bobadilla *et al.*, 2021; Soler *et al.*, 2012; Thaler *et al.*, 2012). Also, investment in plant family specific defence chemistry may be effective against generalists, but ineffective against specialists (Ali & Agrawal, 2012). Recent studies further identify that cross-resistance or susceptibility via induced responses may be highly specific for the interacting herbivore species pair (Mertens *et al.*, 2021a; Moreira *et al.*, 2018). How specificity in response to individual herbivores and physiological constraints of dealing with a multitude of attackers is reflected in plant defence strategies is poorly defined and explored.

Defining plastic defence strategies to match multi-herbivore attack

Here we define the repertoire of plastic defence strategies that may allow plants to use specific responses to individual attackers under a multitude of stressors interacting with the plant (Box 2). A strong specific induced response to a first attacker may limit the potential to respond to other stressors. Several plants show such *canalization* in their response and cannot deploy a full response to subsequent stressors (Viswanathan *et al.*, 2007). The reduced potential to respond to a second attacker may come from prioritizing defence to the first attacker over growth, which reduces energetic potential to mount defences against subsequent attackers (Li *et al.*, 2015; Li *et al.*, 2018). Antagonistic cross-communication between signal transduction pathways may impair resistance to caterpillars after responding to aphid attack (Ali &



Agrawal, 2014; Fernández de Bobadilla *et al.*, 2021; Soler *et al.*, 2012; Thaler *et al.*, 2012). The *canalization* strategy may be adaptive if the first attacker is the most damaging, or if the phenotype in response to this first attacker has broad cross-resistance to subsequent attackers (Adio *et al.*, 2011; Gonzales-Vigil *et al.*, 2011). In these scenarios, plants may save costs of defence since they do not need to invest energy in redirecting resources towards every new stressor. This strategy may not be the optimal response during different ontogenetic stages, or in different plant organs, because it may lead to reduced potential to deal with subsequent attackers or negatively affect interactions with beneficial organisms (Rusman *et al.*, 2019; Thaler *et al.*, 2012). To deal with some of these costs, the strategy can be modified by adopting *tissue specific canalization* where each plant organ particularly responds to the first attacker (Wolinska *et al.*, 2019).

Plants may play a *switching* strategy if resistance to each herbivore requires very different responses, and the costs of switching phenotype are low. The costs not only entail metabolic investment in the new resistance traits, but if the former attacker is still present the switch will lead to vulnerability to the first attacker (Heil, 2010). Thus, completely switching defence phenotype may only be advantageous when multiple herbivores arrive at different moments on the host plant (Barton & Koricheva, 2010; Ochoa-López *et al.*, 2020), or in herbivore-species-poor communities (e.g. high altitude or very dry ecosystems) where it is very rare that two herbivores arrive at the same time. The metabolic costs of switching may be reduced when biosynthetic enzymes and intermediates are shared between pathways that regulate resistance to each individual attacker (Li *et al.*, 2018), for example through remobilization of resources from secondary to primary metabolisms and vice versa or reabsorbing secondary metabolites to synthesize new chemicals needed for a different response. The switching strategy also implies that plants may reconfigure gene networks involved in crosstalk between pathways to avoid antagonistic crosstalk or switch resistance downstream by for example converting secondary chemistry into different



components matching the type of attacker or by resource allocation strategies (Li *et al.*, 2015; Li *et al.*, 2018).

Many plant species find themselves in ecological settings where stressors occur simultaneously including the interaction with several different herbivore species at the same time (Stam *et al.*, 2014). These situations require plants to play a strategy of *integrating* defence responses to all antagonists that may be regulated through crosstalk between signal-transduction pathways. The plant phenotype thus compromises resistance potential to each individual attacker, but yields a net benefit of resistance in perspective of the full attacker community Davidson-Lowe *et al.*, 2019; Eisenring *et al.*, 2018; Mathur *et al.*, 2013; Stam *et al.*, 2017). The costs of an integrated response may arise when plants are not able to effectively deal with the most ravaging herbivore species in terms of their effect on plant fitness. Such costs are likely to be countered by *prioritizing* responses to the most damaging herbivores. Thus, plants may integrate responses to multiple attackers, but upon attack by a key herbivore redirect their resistance to particularly deal with that attacker. The costs arising from prioritizing resistance to one attacker is suboptimal resistance against other attackers and thus such strategies are likely to only arise when fitness costs of attack by herbivore species substantially differ. Under these conditions we may also find plants to strongly respond to particular herbivore species that themselves may not inflict large fitness costs, but with their presence reduce the likelihood that the plant will be colonized by more ravaging herbivores (Poelman & Kessler, 2016). A response to one herbivore may *vaccinate* the plant to arrival of other herbivores and such situations may lead to plants even acquiring some herbivores that are tolerated to reduce costs of herbivory in a community context (Kessler & Baldwin, 2004).

In a community context, plants may play advanced strategies that match predictable patterns of herbivore attack. When arrival of one herbivore species predicts the odds of arrival of subsequent species, plants may evolve *anticipation* strategies (Mertens *et al.*, 2021a,b). In such strategies the induced



response to one herbivore includes a trait expression that prepares for future attack. Oviposition-induced resistance to caterpillar damage (Hilker & Fatouros, 2015; Pashalidou *et al.*, 2015), plants responding to snail cues and becoming more resistant to later arriving insect herbivores (Orrock *et al.*, 2018), or leaf herbivory enhancing resistance to herbivores feeding on reproductive organs (McArt *et al.*, 2013) are examples of this strategy. Anticipatory strategies may only persist under high predictability of herbivore co-occurrence and order of attack (Mertens *et al.*, 2021b).

Playing one or multiple strategies

Adaptiveness of each of these strategies largely depends on community context and it is evident that many plant species are part of communities that are highly stochastic in terms of local dynamics of herbivore and pathogen attack. This not only means that within plant populations frequency-dependent selection may maintain large diversity in (plastic) defence strategies (Davila Olivas *et al.*, 2017), but that individual plants may also be selected to play context-dependent strategies (Mertens *et al.*, 2021b; Hervé *et al.*, 2019). Within one plant the optimal defence strategy to play may depend on attack intensity. This may entail that depending on the severity of attack by a particular herbivore, plants may either integrate a response to multiple attackers at low density of each attacker or prioritize responses to one attacker when its density increases. Per plant organ, plants may play different strategies when leaves are under attack by a large diversity of herbivores, but reproductive tissues only by one specific attacker. The effectiveness of each strategy may also largely differ with plant ontogeny, especially when the costs of defence in trade-off with growth and the likelihood of attack vary across plant age (Barton *et al.*, 2010; Ochoa-Lopez *et al.*, 2020). This entails that individual plants may play ontogeny-determined plastic responses to multi-herbivore attack. Abiotic conditions may limit the potential to deploy particular strategies by resource limitation or by physiological interactions in dealing with abiotic and biotic stress (Nguyen *et al.*, 2016).



BOX 2: Plant strategies

In the context of the multitude of attackers arriving to plants in various ecological settings, a number of defence strategies can be defined. Many of these include advanced risk management strategies that are reflected in physiological responses of plants to herbivory.

Escaping: Plants respond to attackers with an induced shift in growth or phenology that reduces the encounter rate with the attackers (Van Wallendael *et al.*, 2019).

Constitutive defence: Plants express their defences independently of actual herbivore attack (Erb, 2018; Karban, 2020).

General induced response: The induced response against the first attacker is also effective against all subsequent attackers (e.g. cell wall thickening, trichome density, broad spectrum of chemical defences) (Adio *et al.*, 2011; Gonzales-Vigil *et al.*, 2011)

Multifunctional defence traits: Plants use the same compound to defend against different attackers, or with different purposes (Li *et al.*, 2018).

Canalization: The first herbivore attacking the plant has a large impact on the plant defence phenotype. The induced phenotype cannot be changed to respond against subsequent attackers and it determines how the plant interacts with other members of the insect community (Viswanathan *et al.*, 2007).

Switching: The plant has the ability to always adapt the response to the current attacker and tailor the defence phenotype to the identity of the stressor.

Integration: Plants activate a defence response every time a new attacker arrives and integrate this response into a defence phenotype that is effective against the full suite of attackers, but sub-optimal to each individual attacker (Mathur *et al.* 2013; Stam *et al.*, 2017).

Prioritization: Upon damage by the most ravaging herbivore species, plants target their defence to the specific attacker, with a cost of becoming more susceptible to other attackers.

Vaccination: Induced response to a herbivore species that can be tolerated with the benefit of reduced attack by a more ravaging herbivore (Kessler & Baldwin, 2004).

Anticipation: Plants anticipate the arrival of other herbivores based on cues or presence of specific herbivores. In their response to the current attacker, the plant already mobilizes defences for likely forthcoming attack (Mertens *et al.*, 2021a,b; McArt *et al.*, 2013).

Priming: A physiological state of alert in response to environmental cues, that prepares the plant for future attack (Martinez-Medina *et al.*, 2016). The alert state allows plants to rapidly respond to actual attack, not forming the defence in absence of the attacker.



CONCLUDING REMARKS AND FUTURE OUTLOOK

Plant defence strategies against herbivores evolve under complexity of multi-herbivore attack, but have been largely studied in isolation over the past decades. Only very few studies have moved beyond challenging plants with two stressors (Kroes *et al.*, 2016; Mathur *et al.* 2013; Stam *et al.*, 2017), and these studies have only scratched the surface of how plants match their phenotype with natural challenges by a large diversity of herbivore attack (Fernández de Bobadilla *et al.*, 2021). Important challenges are to identify plant limitations to deal with their natural number of stressors to also place our insights in defence plasticity to two attackers in context. In many of the plastic defence strategies we describe here, specific ecological settings may select against antagonistic interactions caused by induced plant responses. For example, when plants are consistently attacked by aphids and subsequently by caterpillars, we may anticipate that plants adapt their plasticity to this situation and reduce the impact of aphid-induced susceptibility to caterpillar attack by reducing antagonism between signalling pathways, correct those in downstream signal transduction or balance energetic costs of defence responses to multiple attackers. This may explain the absence of aphid-induced susceptibility to caterpillar attack in at least some plant species (Ali & Agrawal, 2014; Mertens *et al.*, 2021b; Moreira *et al.*, 2018). Canalization or prioritization strategies may also identify the adaptive nature of antagonistic crosstalk in a community context rather than that this physiological concept may mismatch with the ecological outcome of plant-mediated species interactions.

Providing insights into the physiological strategies and limitations that plants have in dealing with multi-herbivore attack, we may start with uninformed and more artificial, controlled experiments. In these experiments, plants should be challenged with sequential herbivore attack beyond dual attack and address if plants prioritize responses to particular attackers or integrate all challenges into a compromised phenotype. Importantly, such studies should eventually be placed in an ecological context by challenging plants with natural



sequences of herbivore attack potentially in comparison with non-occurring or rare orders of arrival to study what parts of plant plasticity are adaptive to patterns of attack found in nature (Mertens *et al.*, 2021a). At least, we should conclude that in many of the plastic plant defence strategies that we outlined here, the signature of response to an individual stressor is likely to include an adaptation to the community of attackers as a whole. Thus, in transcriptomic studies on “the response of a plant to caterpillar or aphid attack”, we are not just measuring the response to that particular species, but will observe regulation of processes that allow for resistance to other attackers (Mertens *et al.*, 2021b). For example, physiological responses to the focal caterpillar we study include the preparation of the plant for likely pathogen attack in the open wound caused by the caterpillar or prepare the plant for arrival of other insect species that often co-occur with the caterpillar (Davila Olivas *et al.*, 2019). The dependence of plant responses on the plant organ under attack, the density of the attacking herbivore and the time interval between herbivore arrival should be evaluated in the context of the strategies outlined here. This includes identification whether plants may connect local responses to leaf herbivory with anticipation of attack on reproductive organs such as flowers and ripening seeds.

A final challenge is to connect plant physiology and ecology to identify the ecological settings that correspond with the repertoire of defence plasticity of a plant. We should identify whether there is local adaptation by plant populations in defence plasticity to match their specific local pattern of attack, compare plant species in similar ecological context for their plastic defence strategies to multi-herbivore attack and compare related species to understand the macroevolutionary patterns of defence plasticity.



OUTSTANDING QUESTIONS

To what extent does recognition of attack by specific herbivores lead to specificity in plant defence phenotypes?

What are plant limitations in physiological responses to multi-herbivore attack?

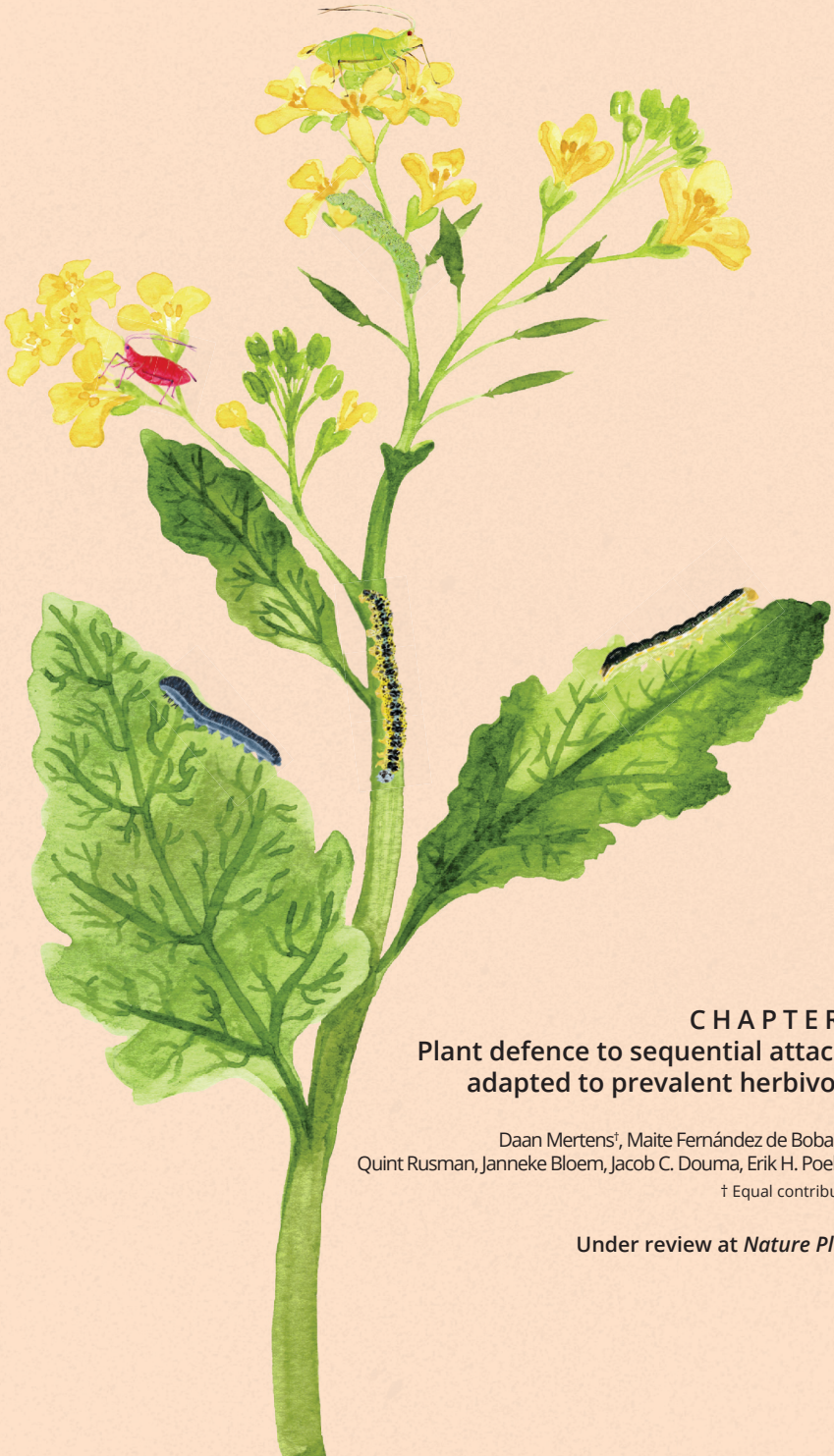
How is antagonistic cross-talk between signal transduction pathways matching multi-stress situations?

Is predictability in the order and timing of attack reflected in plastic plant defence strategies?

Are plants limited in deploying one strategy or can they play strategies depending on the ecological context?

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CHAPTER 3 Plant defence to sequential attack is adapted to prevalent herbivores

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ABSTRACT

Plants have evolved plastic defence strategies to deal with uncertainty of when, by which species and in which order attack by herbivores will take place (Erb & Reymond, 2019; Heil, 2010; Karban, 2020). However, the responses to current herbivore attack may come with a cost of compromising resistance to other, later arriving herbivores. Due to antagonistic cross-talk between physiological regulation of plant resistance to phloem-feeding and leaf-chewing herbivores (Ohgushi, 2005; Poelman *et al.*, 2010; Erb *et al.*, 2012; Soler *et al.*, 2012; Thaler *et al.*, 2012), the feeding guild of the initial herbivore is considered to be the primary factor determining whether resistance to subsequent attack is compromised. We show that, by investigating 90 pair-wise insect-herbivore interactions among ten different herbivore species, resistance of the annual plant *Brassica nigra* to a later arriving herbivore species is not explained by feeding guild of the initial attacker. Instead, the prevalence of herbivore species that arrive on induced plants based on three years of season-long insect community assessments in the field explained cross-resistance. Plants maintained resistance to prevalent herbivores in common patterns of herbivore arrival and compromises in resistance especially occurred for rare patterns of herbivore attack. We conclude that plants tailor induced defence strategies to deal with common patterns of sequential herbivore attack and anticipate arrival of the most prevalent herbivores.



During 400 million years, insect herbivores have been driving the evolution of plant defences (Ehrlich & Raven, 1964; Labandeira & Johnson, 2002). Individual plants are typically attacked by multiple species of insects that arrive at different moments during a plant's lifetime. The occurrence of insect herbivores may be uncertain in terms of when, by which species, and in which order the attack will take place. To save costs of maintaining defences in the absence of herbivores, plants alter their level of defence in response to actual herbivore attack (Erb & Reymond, 2019; Heil, 2010; Karban, 2020). These induced defences also allow plants to tailor resistance to the specific attacker which can be recognized by its damage pattern and compounds in the herbivore's oral secretion (Erb & Reymond, 2019; Erb *et al.*, 2012). However, the specific response to one herbivore may compromise the resistance to other herbivores. Physiological limitations in the regulation of resistance to sequential herbivore attack as well as manipulation of induced plant responses by herbivores may make plants more susceptible to secondary herbivore attack (Ohgushi, 2005; Poelman *et al.*, 2010; Erb *et al.*, 2012; Soler *et al.*, 2012; Thaler *et al.*, 2012).

Although plants are known to rapidly evolve to combinations of species they interact with (Ramos & Schiestl, 2019; Züst *et al.*, 2012), we know surprisingly little about the link between induced responses to herbivory and the odds of sequential herbivore attack that plants may have to deal with. The understanding of plant responses to attack by two sequentially attacking herbivore species is built upon a large collection of studies that arbitrarily selected pairs of herbivores to study plant resistance to sequential attack (Agrawal, 2000; Moreira *et al.*, 2018) and in-depth studies of the underlying molecular mechanisms of plant physiological responses to dual attack (Erb & Reymond, 2019; Voelckel & Baldwin, 2004; Caarls *et al.*, 2015; Proietti *et al.*, 2018). These studies demonstrated that plant responses to attack by leaf-chewing herbivores such as caterpillars are primarily regulated through the jasmonic acid (JA) signalling pathway and generally result in induced resistance to subsequent attack by other leaf chewers (Davidson-Lowe *et al.*, 2019; Eisenring *et al.*, 2018). Plant responses to phloem-feeding herbivores such as aphids, primarily involve the salicylic acid



(SA) signalling pathway and generally result in induced resistance to attack by other phloem-feeding herbivores (Züst & Agrawal, 2016). However, a sequence of phloem-feeding and leaf-chewing herbivores compromises physiological responses to the later arriving species because of antagonistic crosstalk between the SA and JA pathways (Soler *et al.*, 2012; Thaler *et al.*, 2012; Caarls *et al.*, 2015; Proietti *et al.*, 2018; Ali *et al.*, 2014). The current consensus is that plant responses to initial herbivore attack strongly determine the potential to respond to subsequent herbivory and results in compromises of resistance to sequential attack by herbivores of different feeding guilds (Thaler *et al.*, 2012; Moreira *et al.*, 2018). This consensus, based on physiological studies, has largely ignored the apparent negative ecological consequences of induced susceptibility to future herbivory (Poelman *et al.*, 2010; Soler *et al.*, 2012).

Empirical ecological studies and meta-analyses that compared interactions between several pairs of herbivores revealed high specificity in the magnitude of induced resistance or susceptibility in pairwise interactions (Agrawal, 2000; Moreira *et al.*, 2018). In addition to feeding guild, diet breadth in terms of the level of food-plant specialization by herbivores is an important trait determining the nature of induced plant responses as well as the susceptibility of herbivores to the induced plant phenotype (Agrawal, 2000; Bidart-Bouzat & Kliebstein, 2011). Whereas the feeding behaviour and growth of generalist herbivores is strongly affected by food-plant specific classes of chemical defences, specialist herbivores can cope with low concentrations of these compounds but are affected by high concentrations (Ali and Agrawal, 2014). Interestingly, closely related plant species differ in their responses and level of resistance to sequential attack by the same pair of herbivore species (Ali & Agrawal, 2014). The apparent plant-species-specific responses to sequences of attackers indicate differences in selection pressures by sequential herbivory and suggest that plants are not systematically impaired in responding to sequential attack by herbivores of different feeding guilds. We argue that plant responses to initial herbivory should not result in significant compromises in resistance to subsequent attack by the most prevalent and damaging herbivore species. In optimizing their defence



strategy, plants may prepare for likely future attack as part of their induced response to current attack (Mertens *et al.* 2021b).

Our study investigates whether the level of induced resistance to sequential herbivory in the annual plant *Brassica nigra* corresponds with the likelihood of the order and nature of sequential insect attack in the field. We link observations of the order of arrival of insect herbivores during three years of season-long insect community assessment on 488 plants, to a full-factorial assessment of induced resistance in pairwise plant-mediated interactions between ten herbivore species. We specifically test whether induced resistance of the second herbivore can be predicted by traits (feeding guild and diet breadth) of the inducing herbivore or the secondary receiving herbivore species, as well as the commonality of those species and their pairwise interactions in the field (Extended Data Figure 1, Supplementary Information, Table S1)

The ten herbivore species induced species-specific plant responses as characterized by the expression of *LIPXYGENASE 2 (LOX2)* as marker gene for the JA pathway and *PATHOGENESIS-RELATED PROTEIN 1 (PR1)* as a marker for the SA pathway (Table S2) (Appel *et al.*, 2014; Erb & Reymond, 2019). As predicted from the literature (Bidart-Bouzat & Kliebestein, 2011), chewing herbivores induced stronger JA responses compared to phloem feeders indicated by *LOX2* expression levels but weaker SA responses indicated by *PR1* expression levels (Extended Data Figure 2). These differences between feeding guilds were most apparent for specialist herbivores that also induced stronger *LOX2* expression than generalist herbivores (Table S3-S6). However, the traits of the inducing herbivore (feeding guild and diet breadth) did not predict the performance of secondary herbivores feeding on the induced plants (Fig. 1, Table S7- 8). Performance of receiving herbivores was either almost consistently promoted or consistently inhibited on plants previously induced by herbivores, independent of which herbivore species served as inducer (cf. rows in Fig. 1A). Surprisingly, we found much less consistency from the inducing herbivore perspective, as specific inducers could both promote or inhibit performance



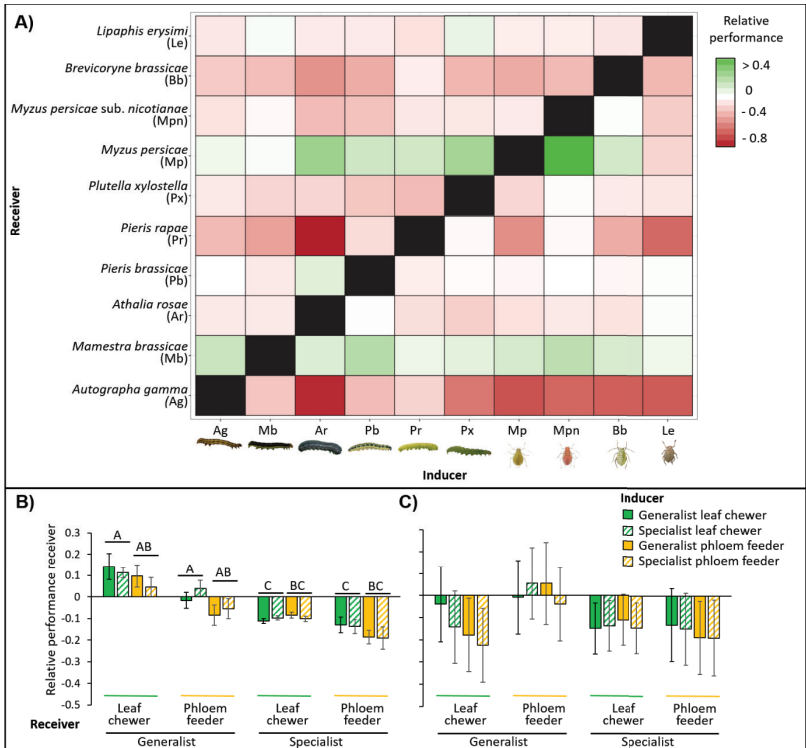


Figure 1. Effect of herbivory by ten primary herbivores (inducers) on the performance of the same ten species as secondary herbivores (receiver) relative to performance on an undamaged control plant. We modelled the performance (number of aphids, or larval weight) of each of the receiving herbivores as a function of the inducing herbivores. We calculated the response ratio for each observation by taking the natural logarithm of the ratio between the observed performance of an individual belonging to each species on a treatment and the modelled mean performance of all individuals of that species on control plants. The log response ratio (LnRR) is a dimensionless measure widely used in meta-analysis²⁶. **(A)** Heat map as visual representation of the effect of each inducer on the relative mean performance of each receiving herbivore. **(B)** and **(C)** show relative performance of receiver based on the feeding guild and diet breadth of the inducer. Bars represent average relative performance \pm SE. Different letters indicate significant differences among treatments in post-hoc analyses (LMM). **(B)** Presents estimations from a model not correcting for inducer identity. **(C)** Corrected for inducer identity.



of subsequent herbivores, even when those were of the same feeding guild (cf. columns in Fig. 1A). Thus, induction by leaf-chewing herbivores did not predict resistance to subsequent feeding by chewers, nor did herbivory by phloem-feeding herbivores predict resistance to subsequent phloem feeders. Moreover, induced responses to phloem feeders did not lead to susceptibility to secondary feeding by chewing herbivores. The plant phenotype induced by one herbivore species resulted in large variation in both sign and magnitude of effect on performance of the nine other secondary herbivore species (Fig. 1A, Extended Data Figure 3). These findings are further supported by the absence of a relation between the induced plant phenotype in terms of *LOX2* and *PR1* expression and secondary herbivore performance (Extended Data Figure 4). Gene expression of *LOX2* and *PR1* upstream in the defence signalling cascade may not predict expression of traits that affect herbivore performance such as primary metabolites, secondary metabolites (glucosinolates), or morphological defences such as trichomes (Supplementary Results and Discussion). Overall, secondary herbivore performance was best explained by diet breadth of the secondary herbivore, but was mostly independent of its feeding guild and largely driven by species-specific effects (Fig. 1B and 1C, Table S7-S10). Where most other studies have evaluated up to ten pairwise interactions, we show by using 90 species interactions (excluding intraspecific interactions) that the identity and/or functional group of the inducing herbivore does not affect the performance of a secondary herbivore on induced plants (Moreira *et al.*, 2018). These findings ask for a shift of focus in our research field, stepping away from the predictive value currently attributed to the identity or functional group of inducing herbivores when inferring an ecological or evolutionary interpretation of induced plant responses to sequential herbivory. Instead, our data clearly show that the attack rates of individual herbivore species in the field are the major determinants of the level of cross-resistance in induced defence strategies.

Based on the assembly of insect communities over the lifetime of 488 plants, we found that five out of the 90 pairwise interactions investigated in our greenhouse experiment were not observed in the field. A total of 23 interactions



occurred on less than 1% and 53 interactions were observed on less than 5% of the 488 plants, while 37 interactions were found on greater than 5% and 11 interactions were observed on more than 25% of the plants (Fig. 2D and Table S12-S14). The frequency of these observed species interactions is determined by overall prevalence of the inducing and secondary herbivore species, as well as by how initial herbivory affects the likelihood of a plant being colonized by a second herbivore species⁵. Our most important finding is that, when assessing the performance of herbivores on previously induced plants relative to their performance on untreated plants in our greenhouse experiment, we observed a reduced performance of specialist herbivores correlated with the increased prevalence of the herbivore under field conditions (Fig. 2A and 2B). This pattern is inverted when interactions involve a generalist secondary herbivore, showing that common interactions (occurring > 3.5% plants) are associated with higher performance of generalist secondary herbivores relative to their performance on non-treated plants (Fig. 2A and 2B). The frequency of interaction pairs to occur was strongly determined by the overall commonality of plant attack by specialist secondary herbivores (Table S11). Irrespective of the identity of the initial herbivore attacking the plant, resistance was strongest against prevalent secondary specialist herbivore species (Extended Data Figure 5). Even if plants were initially attacked by a prevalent phloem-feeding herbivore species, they were not compromised in resistance to prevalent specialist leaf-chewing herbivores. Thus, plant responses to initial herbivory did not compromise resistance to attack by common secondary herbivores (Extended Data Figure 5).

Placing our experimental findings in an ecologically relevant context is imperative for interpretation of the ecology and evolution of induced plant defences. First, the induced response to initial herbivory and enhanced resistance to specialist herbivores is likely adaptive as the insect community on *B. nigra* plants becomes dominated by the most ravaging specialist herbivores as the growing season progresses (Fig. 2C) (Connell, 1980). Moreover, herbivore-induced *Brassica* plants are more frequently colonized by specialist herbivores than undamaged plants (Poelman *et al.*, 2010). Specialist herbivores involved



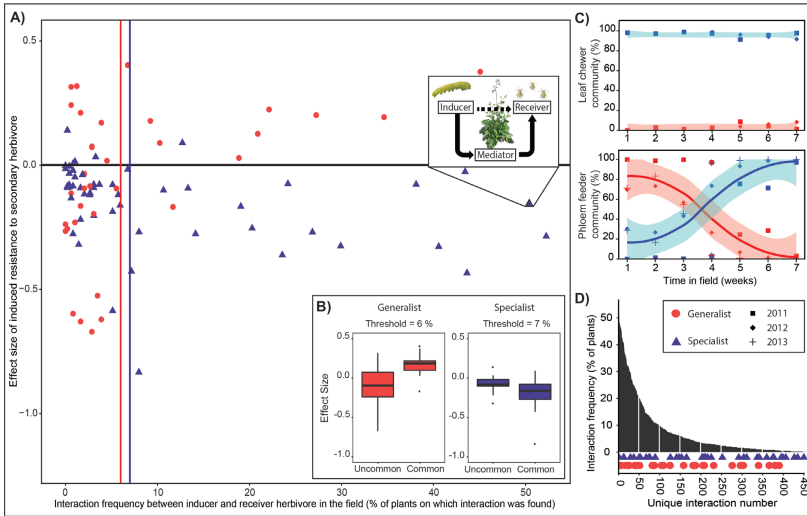


Figure 2. Optimal resistance strategies incorporate likelihood of secondary attack. (A) Outcome of pairwise interactions tested in a greenhouse set-up related to percentage of plants in our field experiment on which these interactions were observed. The coloured vertical lines represent the threshold set in B. (B) Response ratio of pairwise interactions classified as common or uncommon by an interaction frequency threshold. This threshold was set at 6% or 7% of plants in our field depending on whether the secondary herbivore was a generalist (left panel) or a specialist (right panel). These threshold values indicate the percentages of interaction occurrence that yielded clear significant differences between common and uncommon interactions in a sensitivity analysis for 20 threshold values. Response ratio of common and uncommon interactions were significantly different (Mann–Whitney test; generalist $p = 0.004$ and specialist $p = 0.002$). (C) Ratio of specialist and generalist herbivores in the field experiments over time, indicating increasingly specialist dominated herbivore communities. (D) The percentage of plants on which the 453 unique interactions observed in the field occurred. Circles and triangles indicate the interactions we tested in our greenhouse set-up.

in the most common interactions were also the most prevalent herbivores and colonize individual plants with larger numbers than herbivores in rare interactions (Table S13-S14). Together these findings indicate that *B. nigra* plants activate defence strategies to the most likely type of herbivore attack and are not compromised to deal with these attackers by responses to initial herbivory. However, compromises do arise when later arriving herbivores are



rare. Second, we observe apparent maladaptation of induced plant responses to secondary herbivores with a wide diet breadth (generalists) (Fig. 2A and 2B). We propose that the costs of this induced susceptibility are relatively small due to the effectiveness of constitutive resistance (Barton *et al.*, 2010). Even though relative performance of surviving generalist herbivores on induced plants compared to undamaged plants was positive, the mortality rates were high compared to specialist herbivores, and the size of those that survived were small (Extended Data Figure 3). Third, strong negative effects on rare secondary herbivores can be explained by non-mutually exclusive hypotheses (Fig. 2A). They may indicate an evolutionary signature of past arms races between *B. nigra* and the specific herbivore species (Connell, 1980). Alternatively, the strong, induced cross-resistance may indicate resistance to a secondary herbivore that has strong outbreak cycles that did not occur during the three-year period of our field observations (Barbosa *et al.*, 2012). Finally, some of the resistance to uncommon secondary herbivores may be due to trait overlap with secondary herbivore species involved in common interactions and hence similarity in performance on induced plants.

The results of this study demonstrate that *B. nigra* plants optimize their defence responses to herbivory by anticipating the arrival of the most abundant herbivores in response to initial herbivory. To understand the evolution of induced defence strategies, plant physiological responses to single herbivore attack and its plasticity to multi-herbivore attack should thus be evaluated in a community perspective, including the dynamic patterns in order and timing of multi-herbivore attack. The adaptive significance of plant responses to initial attack is largely determined by the predictability of secondary herbivore attack and herbivore traits such as diet breadth. The cross-talk between SA and JA should not be extrapolated to ecological outcomes of herbivore interactions, because substantial variation in plant defence phenotypes may arise after initial expression of genes basal to the JA and SA signal transduction pathways (Züst *et al.*, 2012). SA and JA cross-talk in plants may dampen the overly strong induction of specific defensive phenotypes and allow a fine-tuning where the



urgent need to respond to current stress is traded off to retain the capability to respond to probable subsequent stresses (Thaler *et al.*, 2012). We speculate that initial attack by any herbivore species may result in plants mobilizing resistance to tailor future prevalent herbivores. Thus, the simultaneous upregulation of JA and SA related genes to initial attack found in many studies on induced responses to individual herbivores could presumably be responses targeted to predictable future attack (Mertens *et al.*, 2021b). For instance, *PR1* expression in response to chewers may predict that plants are exposed to a large likelihood of future aphid attack. Hence, plants optimize their defence strategy to match common patterns of sequential herbivore attack, to not compromise responses to the most prevalent herbivore attacks.

METHODS

Study system

Brassica nigra (L.) Koch is an annual herbaceous plant common throughout Europe and used as host plant by a wide range of phytophagous insects (Bischoff & Trémulot, 2011; Schlinkert *et al.*, 2015). Seeds collected from a local population in Wageningen, The Netherlands, were germinated in trays. One-week-old plants were transplanted and grown in pots (ø 15 cm, 1 L) under greenhouse conditions (22 ± 2 °C, 60–70 % r.h. and L16:D8). Four-week-old plants were used in greenhouse experiments, seedlings were planted in field experiments.

Ten insect species were used as herbivores (Table S1). We used first instar larvae of the leaf chewers: the silver Y, *Autographa gamma* (Ag) (Lepidoptera: Noctuidae); the cabbage moth, *Mamestra brassicae* (Mb) (Lepidoptera: Noctuidae); the large cabbage white, *Pieris brassicae* (Pb) (Lepidoptera: Pieridae); the small cabbage white, *P. rapae* (Pr) (Lepidoptera: Pieridae); the turnip sawfly, *Athalia rosae* (Ar) (Hymenoptera: Tenthredinidae); and second instar larvae of the diamondback moth, *Plutella xylostella* (Px) (Lepidoptera: Plutellidae). Starting mass of these newly hatched herbivores is below the error margins of an analytical balance and was not assessed. We used adult wingless individuals of



the phloem feeders: the cabbage aphid, *Brevicoryne brassicae* (Bb); the green peach aphid, *Myzus persicae* (Mp); the tobacco aphid, *Myzus persicae* sub. *nicotianae* (Mpn); and the mustard aphid, *Lipaphis erysimi* (Le) (all Hemiptera: Aphididae). The insects *A. gamma*, *M. brassicae*, *P. brassicae*, *P. rapae*, *B. brassicae* and *P. xylostella* were reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus), while *A. rosae*, *M. persicae*, *M. persicae* sub. *nicotianae* and *L. erysimi*, were reared on radish (*Raphanus sativus*) (all from the stock rearing of the Laboratory of Entomology, Wageningen University). The insect cultures were kept under greenhouse conditions (22 ± 2 °C, 50–70 % r.h. and L16:D8). We classified herbivore species as host specialists when their documented trophic niche in the relevant herbivorous life stages is limited to plants of Brassicaceae (Table S1). The herbivore species were selected for being culturable, the possibility to measure a proxy for their performance (weight gain or population growth) and to represent a balanced spectrum of feeding guilds, level of food plant specialisation and prevalence in the field.

Experimental design

To assess plant defence responses to herbivory and the ecological outcome of all 90 pairwise interactions among the ten different insect herbivore species in our experiment, we divided the experiment in ten blocks. In each experimental block, we used all herbivore species as inducers and only one of the ten species as secondary herbivore (receiver) (Extended Data Figure 1). Each block consisted of 110 plants, with ten plants assigned to each of the herbivore inducer treatments, and ten plants which were left untreated. Treatments were randomly arranged over two benches in a greenhouse compartment (22 ± 2 °C, 60–70 % r.h. and L16:D8). To prevent desiccation of the plants and cross contamination between treatments, we placed plants in inundated trays. We infested the youngest fully developed leaf of each plant with either ten wingless aphids or five first instar larvae. All herbivores were left free to move on the plant, allowing them to choose their preferred feeding sites.



Plant transcriptional responses to herbivore induction

As a measure for plant defensive responses to each of the ten initial herbivores, we quantified levels of transcription of two marker genes in *Brassica nigra*, *LIPOXYGENASE 2 (LOX2)* for the jasmonic acid signalling pathway (JA) (Snoeren *et al.*, 2011) and *PATHOGENESIS-RELATED PROTEIN 1 (PR1)* for the salicylic acid signalling pathway (SA) (Leon-Reyes *et al.*, 2010) (Table S2). In each experimental block, leaf samples were taken at 24 or 96 hours after plants were infested with herbivores. The time points for *LOX2* and *PR1* expression were selected based on their peaks of expression after herbivory in *Brassica nigra* identified in previous studies (Broekgaarden *et al.*, 2011; Fernández de Bobadilla *et al.*, 2021). For each herbivore treatment and time point, we sampled the youngest fully developed leaf on which herbivores had been released from five of the plants. These leaves were always damaged and had the majority of released herbivores still present. Samples were taken by detaching the leaf with a razorblade and punching a leaf disc of 2 cm ϕ with a leaf puncher. Herbivores present on the detached leaf were placed back on the plant. The five leaf discs were combined to constitute one biological replicate, submerged in liquid nitrogen right after sampling, and stored at $-80\text{ }^{\circ}\text{C}$ until further analysis. Per experimental block, a single biological replicate per inducer and time point was obtained, yielding a total of ten biological replicates per treatment and time point for the entire experiment. The frozen samples were ground to a fine powder with a pestle, and RNA was isolated from the plant material by using RNeasy Plant Mini Kit (Qiagen) and treated with DNase I (Invitrogen) following the manufacturer's instructions. After isolation, the RNA concentration was quantified using a NanoDrop ND-100 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA; all samples with OD 260 nm / 280 nm of 1.9–2.2 ratio). Samples were diluted and adjusted to an RNA concentration of 50 ng/ μl . From the RNA samples we synthesized cDNA, using the SensiFAST cDNA synthesis kit (Bioline). We quantified expression levels of each sample by RT-qPCR, using the SensiFAST SYBR no-ROX kit (Bioline). Five ng of the cDNA template were added to the reaction with a total volume of 20 μL . The reactions were performed with shuttle PCR conditions using



a CFX96 Touch™ Real-Time PCR Detection System (Biorad). All reactions were conducted using two technical replicates, and samples were omitted from further analysis if the difference in expression between the technical replicates was higher than 0.5. Plate setups also included negative controls (no template) and inter-run calibrators. Gene-expression data was imported to qBase+ 3.1 (Biogazelle), to calculate Calibrated Normalized Relative Quantity (CNRQ). The CNRQ value represents the relative quantity (gene expression level) of a sample for a given target gene corrected by the expression value of two reference genes for each sample. We tested the following reference genes for expression stability: *ACTIN-2 (ACT2)*, *BETA-TUBULINE (B-TUB)*, *ELONGATION FACTOR-1 (EF1)*, *PEROXIDASE 4 (PER4)*, *SECRETION ASSOCIATED RAS RELATED GTPASE 1A (SAR1A)*, *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE (GAPDH)*. The last two genes were selected as reference genes because they had highest stability (i.e. their expression was not affected by the treatment). Data was corrected for differences between runs using inter-run calibrators.

Herbivore performance

To quantify pairwise plant-mediated interactions among the full matrix of interactions between the ten herbivores (90 interactions, excluding intraspecific interactions), we assessed herbivore performance on herbivore-induced plants. These were the same plants that were sampled for gene expression analyses, allowing for direct correlations between defence gene expression and herbivore performance. Seven days after initial herbivore (inducers) infestation, we removed all herbivores (to exclude direct effects of inducing herbivores on the receiver herbivore and thus isolate plant-mediated effects through herbivore-induced plant responses) and immediately re-infested plants with the second herbivore species (receivers). When removing herbivores, we retrieved inducing herbivores from all plants which were clearly damaged by these herbivores. We placed either 20 wingless aphids or ten first instar larvae of the secondary herbivore (receiving) species on all plants in a block, repeating the setup with a different herbivore receiver in each block. After seven days we assessed the



performance of the receiving herbivore by either counting the number of aphids or by collecting and weighing larvae individually (scales: Sartorius CP2P, Mettler Toledo ML54/01) (Extended Data Figure 1). Aphids were counted twice by different persons, and the average was taken as measure of their performance. Herbivore performance is commonly used as parameter of plant resistance to herbivory and provides insight in how initial herbivory results in induced resistance or susceptibility to secondary herbivores.

Herbivore interaction frequency under field conditions

Information on herbivore interaction frequency under field conditions was collected during three years of common garden experiments (summers of 2011, 2012 and 2013) in an experimental field in Wageningen, The Netherlands. We recorded herbivore identity and time of arrival on a total of 540 *B. nigra* plants (180 plants per year). Insect presence was assessed weekly, surveying herbivore community development on individual plants from seedling until seed maturation. Plants that were monitored less than 4 times were removed from the dataset, retaining 488 plants and 895,236 herbivore observations in the analysis (Table S14). We calculated the relative number of plants on which we recorded one herbivore species arriving before or at the same time as another herbivore species. The 90 pairwise interactions tested in our greenhouse experiments spanned the range from common to rare among all pairwise interactions identified on *B. nigra* plants in the field (Fig. 2D).

Statistical analysis

For each modelling analysis, we selected the best model from a set of candidate models by comparison of the Akaike information criteria (AIC) with a selection threshold of $\Delta 5$ AIC (Johnson *et al.*, 2004). Candidate models differed in the number of fixed factors (and their interactions) and the stochastic distribution. Full models included all interactions between fixed factors and in case mixed models were applied they included all random intercepts. When mixed effect models were applied, we first optimized the random structure, and then the fixed structure of the model (Zuur *et al.*, 2009). When more than



one model was optimal based on our AIC threshold, we explored all optimal models to verify that our interpretation of the results would not differ among models and reported the model with lowest AIC. We further compared the AIC of optimal models with the AIC of null models which did not contain any predictors (i.e. the model assumes the effect of all fixed factors is equal to zero). Parameters of the optimal model were estimated by restricted maximum likelihood estimation or Laplace approximation. After model selection, we evaluated the significance of different factors in the optimal (generalized) linear (mixed) model (see *Plant transcriptional responses to herbivore induction* and *Herbivore performance*). Pairwise post-hoc comparisons were evaluated by Tukey's HSD test and contrasts were considered significantly different at P -values ≤ 0.05 . Statistical analyses were done using the *nlme* (Pinheiro *et al.*, 2019), *lme4* (Bates *et al.*, 2015), *lmerTest* (Zeileis & Hothorn, 2002), and *emmeans* (Lenth, 2018) packages in R (v3.2.4, R Core Team 2016).

Plant transcriptional responses to herbivore induction

To model species-specific effects of herbivore induction on the relative expression of *LOX2* and *PR1* we applied generalized linear models (GLM) with gamma error distribution and log link function. The full models included herbivore species identity and the time point at which the sample was taken (24h or 96h after induction) as explanatory factors. To detect broader patterns based on the feeding behaviour of the herbivores, we performed a second analysis. Here we applied generalized linear mixed models (GLMM) with gamma error distribution and inverse link function to evaluate effects of the inducing herbivore's feeding guild (phloem feeder or chewing herbivore), its level of host specialization (specialist or generalist), and the time point at which the sample was taken (24h or 96h after induction) on the relative expression of *LOX2* and *PR1*. To interpret patterns in transcription levels in terms of feeding guild and host specialization of herbivores while correcting for variable species effects within feeding guild and host specialization, species identity was taken as a random effect. As we were interested in species-specific effects on relative gene expression and exploring patterns on higher functional levels (i.e. herbivore



feeding guild and host specialization) rather than changes in expression over time, we performed pairwise comparisons for the two time points separately.

Herbivore performance

To allow comparison of performance across all herbivore species, we calculated the response ratio (LnRR) for each observation (number of aphids, or larval weight) by taking the natural logarithm of the ratio between the observed performance (X) of an individual (i) belonging to species (S) on treated plants (T), and the mean performance of species (S) on control plants (C):

$$\text{LnRR} = \frac{\text{individual performance on induced plant}}{\text{average species performance on control plants}}$$

$$\text{LnRR} = \ln [X_{\text{IT}} / (X_{\text{SC}})]$$

The response ratio is a practical measure when comparing strength and sign of treatment effects across multiple experiments and different response variables, and is widely used in ecological meta-analysis (Hedges *et al.*, 1999; Lajeunesse, 2011). To get robust estimates of mean performance on control plants, we fitted mixed effect models expressing the herbivore performance (number of aphids or larval weight) as a function of the induction treatment, and including the plant individual and the timepoint at which the leaf sample was taken as random factors. The estimated means on control plants were used as denominator in the response ratios. We applied linear mixed effect models (LMM) for each of the phloem-feeding herbivores and *Pieris brassicae*, and generalized linear mixed models (GLMM) with gamma error distribution and log link function for each of the remaining leaf chewing herbivores. We found heterogeneity of variance in the models estimating phloem feeder performance and in the model estimating *P. brassicae* performance. To adjust for this, we allowed variance to be different for each of the inducing herbivores.

Next, we evaluated if herbivore performance depended on the inducing or receiving herbivore. We applied a linear mixed effect model (LMM) with



response ratio as dependent variable and inducing and receiving herbivore identity as independent variables. We corrected for dependency between observations by including the plant individual as random intercept and adjusted for heterogeneity of variance by allowing variance to be different for each of the receiving species. The alternative model that was tested was a model that related the response ratio to the feeding guild (phloem feeder or chewing herbivore) and level of host specialization (specialist or generalist) of the inducer and receiver herbivores with plant individual as random effect. We excluded pairwise interactions between the herbivores themselves, and between *Myzus persicae* sub. *nicotianae* and *M. persicae* from this analysis, as for the latter the data obtained was an outlier in effect size. Although including this interaction did not significantly change the interpretation of our results, it was influential in determining confidence intervals and AIC values, hampering convergence of analysis of the full matrix of interactions. In a third analysis we added a random effect including species identity of both the inducing and receiving herbivore to the model evaluating feeding guild and level of host plant specialization.

In a final analysis we assessed the role of prevalence of both the inducing and receiving herbivores as observed under field conditions in predicting the performance of the receiving herbivore. In these models, the estimated response ratios of pairwise interactions was included as dependent variable, and the feeding guild (phloem feeder or chewing herbivore), level of host specialization (specialist or generalist) and prevalence of the inducer and receiver herbivores were included as explanatory variables. The backward selection procedure applied to optimize the model converged on an optimum that included a complex four-way interaction. Hence, to avoid overinterpretation of our data, we chose to optimize the model by applying a forward stepwise selection procedure.

Relation to field observations

To evaluate whether patterns in herbivore performance under greenhouse conditions could be linked to interaction frequency under field conditions, we related the frequency of each tested pairwise interaction observed in the field



to their estimated mean response ratio in the performance assays. We annotated interactions based on host specialization of the receiving herbivore (host specialists or generalists), and analysed the two groups separately. We classified interactions as either common or uncommon for a range of 20 threshold values, based on the percentage of plants on which interactions were observed (i.e. interaction frequency). To statistically compare the mean response ratio of common and uncommon interactions at each of the 20 threshold values, we applied a non-parametric rank-based test (Mann-Whitney). To assess variability of the observed effect size independent of the sign of the effect, we transformed the estimated mean response ratio by taking its absolute value. This transformed dataset was subjected to the same non-parametric analysis with 20 threshold values of interaction frequencies used in our previous analysis. As the number of common or uncommon interactions was dependent on the assessed interaction frequency threshold, we compared the variance of the group with the least number of pairwise interactions with the variance of an equal number of randomly selected interactions from the largest group. This permutation procedure was repeated 1,000 times for each assessed threshold level. We used prevalence of herbivores (the number of plants colonized by a specific species) as a measure for the importance of interactions, instead of the abundance of the herbivore in the field (number of individuals counted over all plants). Abundance of herbivores on an individual plant may not accurately compare fitness costs of herbivory across species, since the costs of attack by a large number of aphids may not compare to attack by a single ravaging caterpillar.

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AUTHOR CONTRIBUTIONS EHP, DM and MFB planned and designed the study and developed the methodology; EHP, DM, MFB, QR and JB performed the experiments; MFB and JB analysed gene expression; DM and BD analysed performance and field data; all authors contributed to writing of the manuscript;

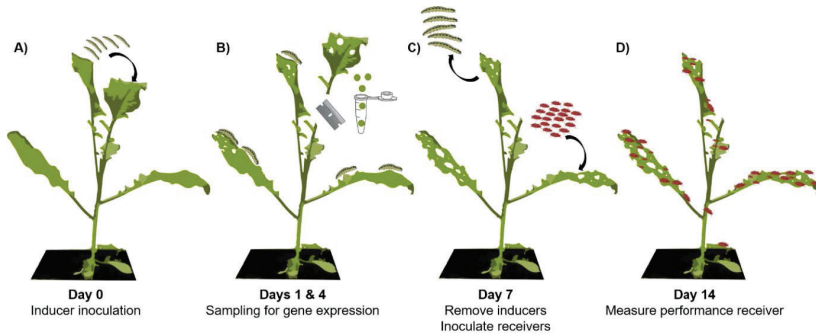
COMPETING INTERESTS Authors declare no competing interests;

DATA AND MATERIALS AVAILABILITY All data is available in the main text or the supplementary materials.

ADDITIONAL INFORMATION Supplementary Information is available for this paper.

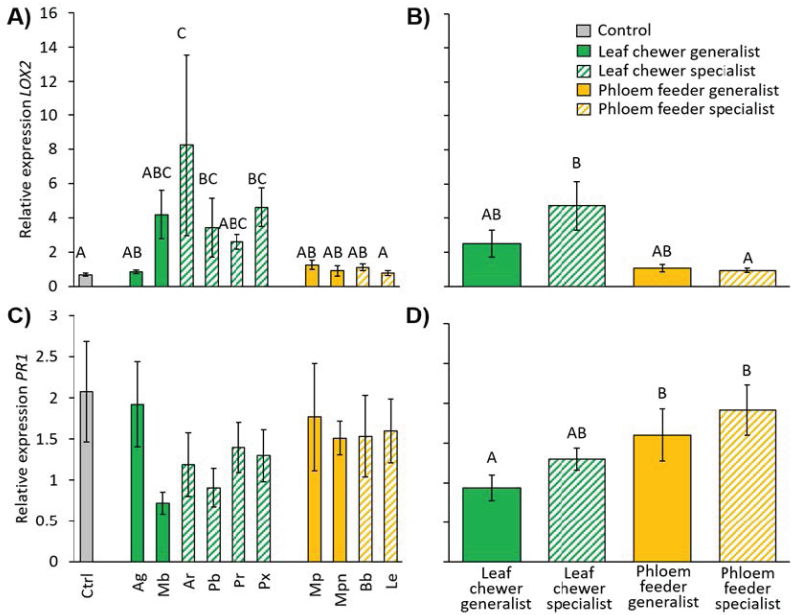


EXTENDED DATA FIGURES



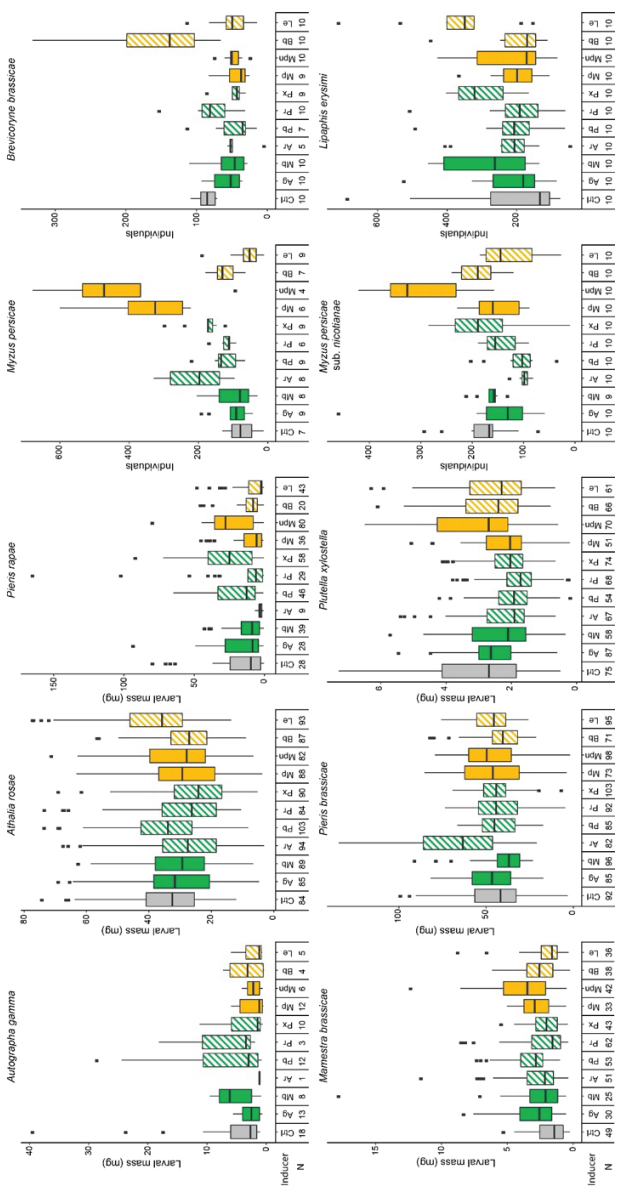
Extended Data Figure 1. Experimental setup for the performance experiment. A) *Brassica nigra* plants were infested with one of ten insect species as primary herbivores (inducers). Five leaf chewers or ten phloem feeders were used as inducers. B) One and four days after plant infestation with herbivores, leaf samples for gene expression analyses were taken (each plant was sampled only once). C) Seven days after plant infestation with herbivores, all remaining inducers were removed and plants were infested with secondary herbivores (receivers). Ten leaf chewers or 20 phloem feeders were used as receivers. D) Seven days after plant infestation with receivers, their performance was measured (leaf chewer weight or number of phloem feeders). We repeated this setup ten times, each time using a different insect as receiver, and preparing ten plant replicates per treatment (Table S1).



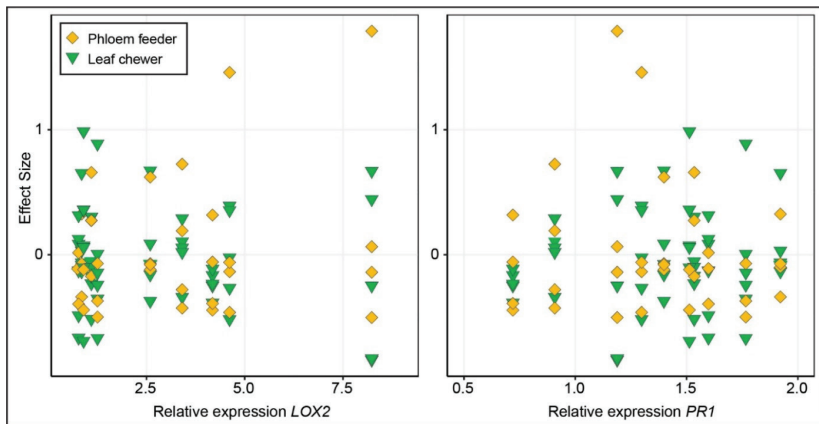


Extended Data Figure 2. Expression levels of the JA biosynthesis gene *LOX2* and the SA responsive gene *PR1* on *Brassica nigra* leaves after 96h of herbivory. Herbivores used were the leaf chewers *Autographa gamma* (Ag), *Mamestra brassicae* (Mb), *Athalia rosae* (Ar), *Pieris brassicae* (Pb), *P. rapae* (Pr) and *Plutella xylostella* (Px), and the phloem feeders *Myzus persicae* (Mp), *M. persicae* sub. *nicotianae* (Mpn), *Brevicoryne brassicae* (Bb) and *Lipaphis erysimi* (Le). **A)** Effects of herbivory on the relative expression of *LOX2* for each herbivore species. **B)** Effects of herbivory on the relative expression of *LOX2* for herbivores grouped by feeding guild and diet breadth. **C)** Effects of herbivory on the relative expression of *PR1* for each herbivore species. **D)** Effects of herbivory on the relative expression of *PR1* for each herbivore species grouped by feeding guild and diet breadth. Bars represent mean \pm SE. Different letters refer to significant differences at $P < 0.05$ based on GLM and Tukey HSD tests (Table S3).



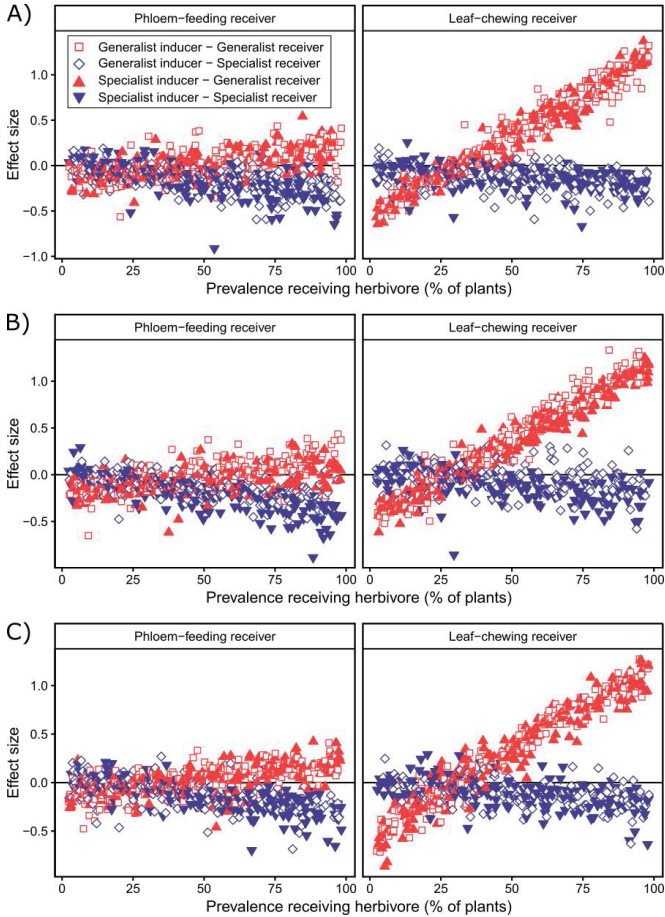


Extended Data Figure 3. Performance of receiving secondary herbivores on *Brassica nigra* plants induced by one of ten different herbivore species. Larval mass or number of individuals on plants induced by herbivory from the leaf chewers *Autographa gamma* (Ag), *Mamestra brassicae* (Mb), *Athalia rosae* (Ar), *Pieris brassicae* (Pb), *P. rapae* (Pr) and *Plutella xylostella* (Px), and from the phloem feeders *Myzus persicae* (Mp), *M. persicae* sub. *nicotianae* (Mpn), *Brevicoryne brassicae* (Bb) and *Lipaphis erysimi* (Lc), and on non-treated plants (Ctrl). Panels correspond to the different receiving herbivores and the numbers below indicate the recapture rate for chewing herbivores, or the number of plants on which phloem feeder populations were assessed.



Extended Data Figure 4. Performance of herbivores is not explained by marker gene expression. Performance of receiving herbivores (effect size; the natural logarithm of the ratio between individual performance on induced plant / average species performance on control plants) related to the relative expression of the JA-marker gene *LOX2* (left panel) or the SA-marker gene *PR1* (right panel). Colours and shapes correspond to the feeding guild of the receiving herbivore.





Extended Data Figure 5. Resistance strategies are predicted by prevalence of the receiving herbivore. Model predictions of performance of receiving herbivores (effect size; the natural logarithm of the ratio between individual performance on induced plant / average species performance on control plants) related to the prevalence of the receiving herbivore, separated for phloem-feeding herbivores (left panels) and leaf-chewing herbivores (right panels). Colours and shapes correspond to combinations of diet breadth of the inducing and receiving herbivores. **A)** Model predictions did not constrain the prevalence of the inducing herbivore, **B)** predictions were constrained in prevalence of the inducing herbivore at 2.5% of plants. **C)** predictions were constrained in prevalence of the inducing herbivore at 50% of plants.



Table S1. Insects used in the greenhouse experiments, their diet breadth and feeding guild. In brackets abbreviations used for species identification in the text and figures.











	Species (Abbreviation)	Diet breath	Feeding guild
	<i>Autographa gamma</i> (AG)	Generalist	Leaf chewer
	<i>Mamestra brassicae</i> (Mb)	Generalist	Leaf chewer
	<i>Athalia rosae</i> (Ar)	Specialist	Leaf chewer
	<i>Pieris brassicae</i> (Pb)	Specialist	Leaf chewer
	<i>Pieris rapae</i> (Pr)	Specialist	Leaf chewer
	<i>Plutella xylostella</i> (Px)	Specialist	Leaf chewer
	<i>Myzus persicae</i> (Mp)	Generalist	Phloem feeder
	<i>Myzus persicae</i> sub. <i>nicotianae</i> (Mpn)	Generalist	Phloem feeder
	<i>Brevicoryne brassicae</i> (Bb)	Specialist	Phloem feeder
	<i>Lipaphis erysimi</i> (Le)	Specialist	Phloem feeder

Table S2. Four genes used in qRT-PCR to quantify defensive responses of *Brassica nigra* to herbivory.

Target	Full name	Pathway	Forward sequence (5'-3')	Reverse sequence (5'-3')
LOX2	LIPOXYGENASE	JA	TGCTCGTGACAGC-CAGAGTC	AGCCAGCCCCCT-GCTGATGA
PR-1	PATHOGENESIS-RELATED PROTEIN 1	SA	CGCCGACGGAC-TAAGAGGCG	ACACCTCGCTTTC-CACATCCA
SAR1A	SECRETION ASSOCIATED RAS RELATED GTPASE 1A	Reference gene	ATCTCTAGCCACCG-TTCCT	TTCTGACGATGCT-GCACAT
GAPDH	GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE	Reference gene	GGAGCTGCCAAG-GCTGTCGG	CCTTCAGA-TTCCTCCTTGATA-GCC



Table S3. Effects of the inducing herbivore's identity and time of sampling (24h or 96h) on relative expression of *LOX2* of *Brassica nigra* plants. **A.** Main model selection steps indicating AIC values of the full model (including all factors and their interaction), the null model (assuming all factors equal to zero), and the optimal model (lowest AIC value). **B.** Results from the optimal model (generalized linear model (GLM) with gamma error distribution and log link function). Values in bold denote significant factors ($P < 0.05$).

A.			
Model	Expression	AIC	
Full	<i>LOX2</i> ~ Inducing Herbivore * Time	633	
Optimal	<i>LOX2</i> ~ Inducing Herbivore + Time	626	
Null	<i>LOX2</i> ~ 1	761	

B.			
Fixed factor	χ^2	df	<i>P</i>
Inducing Herbivore	140.18	10	< 0.001
Time	11.602	1	< 0.001

Table S4. Effects of herbivore feeding guild (phloem feeder or leaf chewer), diet breadth (host generalist or specialist), and time of sampling (24h or 96h) on expression of *LOX2* of *Brassica nigra* plants. **A.** Main model selection steps indicating AIC values of the full model (including all fixed factors, their interactions, and all random factors), the null model (assuming all fixed factors equal to zero), and the optimal model (lowest AIC value). We adjusted for species-specific effects by including herbivore identity as random intercept. **B.** Results from the optimal model (generalized linear mixed model (GLMM) with gamma error distribution and inverse link function). Values in bold denote significant factors ($P < 0.05$).

A.			
Model	Expression	AIC	
Full	<i>LOX2</i> ~ Guild * Specialization * Time + (1 Inducing Herbivore)	622	
Optimal	<i>LOX2</i> ~ Guild * Specialization + Time + (1 Inducing Herbivore)	616	
Null	<i>LOX2</i> ~ 1 + (1 Inducing Herbivore)	640	

B.			
Fixed factor	χ^2	df	<i>P</i>
Guild	22.952	1	< 0.001
Specialization	3.101	1	0.078
Time	17.071	1	< 0.001
Guild * Specialization	4.5	1	0.034



Table S5. Effects of the inducing herbivore's identity and time of sampling (24h or 96h) on expression of *PR1* of *Brassica nigra* plants. Main model selection steps indicating AIC values of the full model (including all factors and their interaction) and the null model (assuming all factors equal to zero). The null model (generalized linear model (GLM) with gamma error distribution and log link function) resulted in lowest AIC score.

Model	Expression	AIC
Full	$PR1 \sim \text{Inducing Herbivore} * \text{Time}$	726
Null	$PR1 \sim 1$	697

Table S6. Effects of herbivore feeding guild (phloem feeder or leaf chewer), diet breadth (host generalist or specialist), and time of sampling (24h or 96h) on expression of *PR1* of *Brassica nigra* plants. **A.** Main model selection steps indicating AIC values of the full model (including all fixed factors and their interactions, and all random factors), the null model (assuming all fixed factors equal to zero), and the optimal model (lowest AIC value). We adjusted for species specific effects by including the herbivore identity as random intercept. **B.** Results from the optimal model (generalized linear mixed model (GLMM) with gamma distribution and inverse link function). Values in bold denote significant factors ($P < 0.05$).

A.

Model	Expression	AIC
Full	$PR1 \sim \text{Guild} * \text{Specialization} * \text{Time} + (1 \text{Inducing Herbivore})$	510
Optimal	$PR1 \sim \text{Guild} * \text{Time} + \text{Specialization} + (1 \text{Inducing Herbivore})$	505
Null	$PR1 \sim 1 + (1 \text{Inducing Herbivore})$	508

B.

Fixed factor	χ^2	df	P
Guild	1.954	1	0.162
Specialization	2.455	1	0.937
Time	0.006	1	0.117
Guild * Time	7.021	1	0.008



Table S7. Effects of the inducing and receiving herbivore species' identities on performance (LnRR) of the receiving herbivore in our greenhouse experiments. **A.** Main model selection steps indicating AIC values of the full model (including all fixed factors and their interactions, and all random factors) and the null model (assuming all fixed factors equal to zero). We adjusted for dependency of observations by including plant identity (Plant ID) as random intercept. **B.** Results from the full model (linear mixed effect model (LMM)). Values in bold denote significant factors ($P < 0.05$).

A.			
Model	Expression	AIC	
Full	$\text{LnRR} \sim \text{Inducing Herbivore} * \text{Receiving Herbivore} + (1 \text{Plant ID})$	- 488	
Null	$\text{LnRR} \sim 1 + (1 \text{Plant ID})$	1323	

B.			
Fixed factor	<i>F</i>	df	<i>P</i>
Inducing Herbivore	4.189	9	< 0.001
Receiving Herbivore	58.637	9	< 0.001
Inducing Herbivore * Receiving Herbivore	3.985	81	< 0.001



Table S8. Performance of herbivores on *Brassica nigra* plants induced by different herbivores in a greenhouse experiment. To allow comparison across multiple receiving herbivore species, we transformed the data by taking the natural logarithm of the ratio between the performance (number of aphids, or larval weight) of herbivores on induced plants and its mean performance on uninfested control plants (LnRR). Performance for each pairwise interaction is estimated by applying a linear mixed effect model (LMM). Interaction frequency represents the percentage of *B. nigra* plants in the field experiments on which the inducing herbivore species was observed before or at the same time as the receiving herbivore species. Two interactions were excluded (Mpn – Mp and Ar – Ag; annotated with an asterisk) due to uncertainty about the data and extremely low recapture rates respectively. Abbreviations: Ag, *Autographa gamma*; Ar, *Athalia rosae*; Bb, *Brevicoryne brassicae*; Le, *Lipaphis erysimi*; Mb, *Mamestra brassicae*; Mp, *Myzus persicae*; Mpn, *Myzus persicae* sub. *nicotianae*; Pb, *Pieris brassicae*; Pr, *Pieris rapae*; Px, *Plutella xylostella*.

Inducer	Receiver	Mean performance (LnRR)	SE	Interaction frequency (% of plants)
Px	Bb	-0.2876	0.0729	52.25
Mp	Px	-0.1575	0.0432	50.41
Px	Mp	0.3764	0.0775	45.08
Mp	Pr	-0.4356	0.0939	43.65
Px	Pr	-0.0270	0.0758	43.44
Mp	Bb	-0.3289	0.0729	40.57
Bb	Px	-0.0781	0.0400	38.11
Bb	Mp	0.1931	0.0879	34.63
Bb	Pr	-0.3269	0.1217	29.92
Pr	Mp	0.2014	0.0949	27.25
Pr	Px	-0.2708	0.0398	26.84
Pr	Bb	-0.0758	0.0691	24.18
Mb	Pr	-0.3633	0.0902	23.57
Mp	Mb	0.2240	0.0597	22.13
Px	Mb	0.1260	0.0520	20.90
Mb	Bb	-0.2550	0.0691	20.29
Mb	Px	-0.1671	0.0409	19.06
Mb	Mp	0.0284	0.0822	18.85
Le	Bb	-0.2777	0.0691	14.14
Le	Px	-0.0935	0.0406	13.32



Table S8. Continued.

Inducer	Receiver	Mean performance (LnRR)	SE	Interaction frequency (% of plants)
Px	Le	0.0897	0.0668	12.70
Le	Mp	-0.1691	0.0775	11.68
Bb	Le	-0.1016	0.0668	10.66
Pr	Mb	0.0890	0.0474	10.25
Bb	Mb	0.1780	0.0560	9.22
Ar	Pr	-0.8361	0.1808	8.20
Mpn	Bb	-0.2702	0.0691	7.99
Ar	Bb	-0.4290	0.0978	7.17
Ar	Mp	0.4033	0.0822	6.76
Mpn	Px	-0.0178	0.0396	6.76
Ar	Px	-0.1623	0.0407	5.94
Mp	Ar	-0.1199	0.0374	5.74
Px	Mpn	-0.0945	0.0654	5.53
Mpn	Mp	0.6654	0.1162	* 5.53
Le	Pr	-0.5880	0.0861	5.12
Mp	Le	-0.0795	0.0668	5.12
Px	Ar	-0.1881	0.0373	5.12
Bb	Mpn	0.0175	0.0654	4.51
Ar	Mb	0.1709	0.0502	3.89
Bb	Ag	-0.6231	0.2269	3.89
Mpn	Le	-0.0786	0.0668	3.69
Px	Ag	-0.5272	0.1438	3.48
Mb	Le	0.0328	0.0668	3.28
Ag	Bb	-0.2037	0.0691	3.07
Ag	Px	-0.0785	0.0383	3.07
Le	Mpn	-0.1971	0.0654	3.07
Mb	Ar	-0.0916	0.0377	3.07
Ag	Mp	0.0721	0.0775	2.87



Table S8. Continued.

Inducer	Receiver	Mean performance (LnRR)	SE	Interaction frequency (% of plants)
Le	Mb	0.0748	0.0554	2.87
Mp	Ag	-0.6730	0.1319	2.87
Bb	Ar	-0.1128	0.0380	2.66
Pr	Ar	-0.1209	0.0382	2.66
Mp	Mpn	-0.0850	0.0654	2.66
Mb	Mpn	-0.0354	0.0690	2.05
Pr	Le	-0.1176	0.0668	2.05
Pr	Mpn	-0.0942	0.0654	2.05
Mpn	Pr	-0.0389	0.0677	1.84
Ag	Le	-0.0923	0.0668	1.64
Le	Ag	-0.6313	0.2025	1.64
Pb	Mp	0.2110	0.0775	1.64
Pb	Px	-0.2200	0.0416	1.64
Pr	Ag	-0.1648	0.2630	1.64
Pb	Bb	-0.3210	0.0826	1.43
Mpn	Mb	0.3184	0.0533	1.23
Bb	Pb	-0.0487	0.0363	1.02
Le	Ar	0.0144	0.0381	1.02
Mb	Ag	-0.2315	0.1604	1.02
Ag	Pr	-0.2777	0.1062	0.82
Ar	Le	-0.0852	0.0668	0.82
Le	Pb	0.0054	0.0343	0.82
Mpn	Ag	-0.5998	0.1832	0.82
Px	Pb	-0.0219	0.0334	0.82
Ag	Mb	0.2419	0.0595	0.61
Ag	Mpn	-0.1140	0.0654	0.61
Pb	Mb	0.3155	0.0488	0.61
Pb	Pr	-0.1334	0.0841	0.61



Table S8. Continued.

Inducer	Receiver	Mean performance (LnRR)	SE	Interaction frequency (% of plants)
Mp	Pb	-0.0341	0.0364	0.41
Mpn	Ar	-0.0892	0.0392	0.41
Mpn	Pb	-0.0051	0.0336	0.41
Pb	Le	-0.0838	0.0668	0.41
Pr	Pb	-0.0801	0.0339	0.41
Ag	Ar	-0.0915	0.0388	0.20
Ar	Mpn	-0.2575	0.0654	0.20
Ar	Pb	0.1402	0.0344	0.20
Mb	Pb	-0.0858	0.0336	0.20
Ag	Pb	-0.0022	0.0342	0.00
Pb	Ag	-0.2666	0.1329	0.00
Pb	Ar	-0.0151	0.0368	0.00
Pb	Mpn	-0.2393	0.0654	0.00
Ar	Ag	-0.7962	-	* 0.00



Table S9. Main steps for selecting the appropriate random intercepts in linear mixed effect models (LMM) testing the effect of inducer and receiver feeding guilds and host specialization on performance (LnRR) of the receiving herbivore. We further corrected for dependency of observations by including plant identity (Plant ID) as random intercept. Large changes in AIC values indicate increase in fit of the model when random intercepts were added or removed from the models. The null model resulted in lowest AIC score. GI = Guild inducing herbivore, SI = Specialization inducing herbivore, GR = Guild receiving herbivore, SR = Specialization receiving herbivore.

Model	Expression	AIC
Full model	$\text{LnRR} \sim \text{GI} * \text{SI} * \text{GR} * \text{SR} + (1 \text{Plant ID}) + (1 \text{Inducing herbivore}) + (1 \text{Receiving herbivore})$	806
Adjusted for inducer	$\text{LnRR} \sim \text{GI} * \text{SI} * \text{GR} * \text{SR} + (1 \text{Plant ID}) + (1 \text{Inducing herbivore})$	1062
Adjusted for receiver	$\text{LnRR} \sim \text{GI} * \text{SI} * \text{GR} * \text{SR} + (1 \text{Plant ID}) + (1 \text{Receiving herbivore})$	812
Not adjusted for species	$\text{LnRR} \sim \text{GI} * \text{SI} * \text{GR} * \text{SR} + (1 \text{Plant ID})$	1064
Null model	$\text{LnRR} \sim 1 + (1 \text{Plant ID}) + (1 \text{Inducing herbivore}) + (1 \text{Receiving herbivore})$	789



Table S10. Effects of the inducing and receiving herbivore feeding guilds and host specialization on performance (LnRR) of the receiving herbivore, not adjusted for species identities. **A.** Main model selection steps indicating AIC values of the full model (including all fixed factors and their interactions, and all random factors) and the null model (assuming all fixed factors equal to zero). We adjusted for dependency of observations by including plant identity (Plant ID) as random intercept. We accounted for heterogeneity of variance by allowing different variance for the different levels of the inducer guild, and specialisation of the receiving herbivore. GI = Guild inducing herbivore, SI = Specialization inducing herbivore, GR = Guild receiving herbivore, SR = Specialization receiving herbivore. **B.** Results from the optimal model (linear mixed effect model (LMM)). Values in bold denote significant factors ($P < 0.05$).

A.			
Model	Expression	AIC	
Full	$\text{LnRR} \sim \text{GI} * \text{SI} * \text{GR} * \text{SR} + (1 \text{Plant ID})$	1098	
Optimal	$\text{LnRR} \sim \text{GI} * \text{SR} + (1 \text{Plant ID})$	1032	
Null	$\text{LnRR} \sim 1 + (1 \text{Plant ID})$	1058	

B.			
Fixed factor	χ^2	df	P
Guild inducing herbivore	0.9910	9	0.319
Specialisation receiving herbivore	44.7509	9	< 0.001
Guild inducing herbivore * Specialisation receiving herbivore	3.3179	81	< 0.069



Table S11. Effects of the inducer and receiver feeding guilds, host specialization, and prevalence in the field on performance (LnRR) of the receiving herbivore. We further corrected for dependency of observations by including plant identity (Plant ID) as random intercept. **A.** Main model selection steps indicating AIC values of the null model (assuming all fixed factors equal to zero), the optimal model, and an overview of the contribution in terms of AIC of fixed factors to the model fit. The full model including all explanatory factors did not converge due to overfitting. GI = Guild inducing herbivore, SI = Specialization inducing herbivore, PI = Prevalence inducing herbivore, GR = Guild receiving herbivore, SR = Specialization receiving herbivore, PR = Prevalence receiving herbivore. We accounted for heterogeneity of variance by allowing different variance for the different levels of feeding guild in the inducing herbivore, and by allowing variance to exponentially increase with increasing receiver prevalence. **B.** Results from the optimal model. Values in bold denote significant factors ($P < 0.05$).

A.			
Model	Expression	AIC	
Null model	LnRR ~ 1	565	
Optimal model	LnRR ~ GR * PI + SR * GR * RP + PR * PI * SI	293	
- Interaction (GR * PI)	LnRR ~ SR * GR * RP + PR * PI * SI	308	
- Interaction (SR * GR * RP)	LnRR ~ GR * PI + SR * GR + GR * RP + SR * RP + PR * PI * SI	349	
- Interaction (PR * PI * SI)	LnRR ~ GR * PI + SR * GR * RP + PR * PI + PI * SI + PR * SI	297	
B.			
Fixed factor	χ^2	df	P
GR * PI	16.9702	1	< 0.001
SR * GR * RP	59.9579	1	< 0.001
PR * PI * SI	6.5973	1	0.010



Table S12. Results of the permutation analysis testing the hypothesis that the variance in receiver performance of uncommon plant-mediated herbivore-herbivore interactions is larger than the variance in performance of common interactions. The data was transformed by taking the absolute value of the ln response ratio of the performance (number of aphids or larval weight). Interactions were classified as common or uncommon based on the percentage of *Brassica nigra* plants on which they occurred in the field experiments. At each assessed threshold value, we calculated the variance for the group (common or uncommon) with the least number of interactions, and compared it with the variance of an equal number of randomly selected interactions from the group with most interactions. This random permutation procedure was repeated 1,000 times for each assessed threshold value. The confirmation rate is the percentage of permutations for which the variance of uncommon interactions is larger than the variance of common interactions.

Threshold (% of plants)	Generalist receiver			Specialist receiver			MAN - U	P
	N uncommon interactions	N common interactions	MAN - U	N uncommon interactions	N common interactions	MAN - U		
50	34	0	-	-	2	52	26	0.2739
40	1	33	32	0.1176	5	49	67	0.102
30	2	32	56	0.0891	6	48	100	0.2378
25	3	31	80	0.0411	8	46	104	0.0517
20	5	29	123	0.0115	11	43	133	0.0255
15	6	28	136	0.017	12	42	139	0.0178
10	8	26	154	0.0428	16	38	198	0.0449
9	9	25	170	0.0241	16	38	198	0.0449
8	9	25	170	0.0241	17	37	182	0.0129
7 *	9	25	170	0.0241	19	35	162	0.0016
6 *	10	24	194	0.0041	20	34	188	0.0059
5	11	23	197	0.0084	25	29	170	0.0006



Table S12. Continued.

Threshold (% of plants)	Generalist receiver			Specialist receiver		
	N uncommon interactions	N common interactions	MAN - U P	N uncommon interactions	N common interactions	MAN - U P
4.5	12	22	203 0.0096	25	29	170 0.0006
4	12	22	203 0.0096	25	29	170 0.0006
3.5	14	20	203 0.0273	26	28	181 0.0012
3	16	18	187 0.1441	30	24	202 0.0054
2.5	20	14	171 0.2901	32	22	186 0.003
2	22	12	160 0.3267	33	21	177 0.0022
1.5	25	9	130 0.5137	36	18	157 0.0018
1	27	7	118 0.3351	39	15	143 0.0033
0.5	31	3	78 0.0582	44	10	92 0.0034



Table S13. Results of the permutation analysis testing the hypothesis that the variance in receiver performance of uncommon plant-mediated herbivore-herbivore interactions is larger than the variance in performance of common interactions. The data was transformed by taking the absolute value of the ln response ratio of the performance (number of aphids or larval weight). Interactions were classified as common or uncommon based on the percentage of *Brassica nigra* plants on which they occurred in the field experiments. At each assessed threshold value, we calculated the variance for the group (common or uncommon) with the least number of interactions, and compared it with the variance of an equal number of randomly selected interactions from the group with most interactions. This random permutation procedure was repeated 1.000 times for each assessed threshold value. The confirmation rate is the percentage of permutations for which the variance of uncommon interactions is larger than the variance of common interactions.

Threshold (% of plants)	N uncommon interactions	Estimated variance uncommon interactions	N common interactions	Estimated variance common interactions	Confirmation rate (%)
50	2	0.0274	86	0.0299	50.5
40	6	0.0310	82	0.0296	47.1
30	8	0.0301	80	0.0302	57.5
25	11	0.0311	77	0.0311	74.1
20	16	0.0331	72	0.0325	88.7
15	18	0.0333	70	0.0331	91.4
10	24	0.0352	64	0.0350	97.9
9	25	0.0363	63	0.0361	98.8
8	26	0.0301	62	0.0298	61.7
7	28	0.0297	60	0.0296	63.7
6	30	0.0293	58	0.0293	58.1
5	36	0.0293	52	0.0290	55.8
4.5	37	0.0292	51	0.0291	57.0
4	37	0.0292	51	0.0291	56.4
3.5	40	0.0262	48	0.0264	0.00
3	46	0.0263	42	0.0312	10.1
2.5	52	0.0225	36	0.0334	6.90
2	55	0.0239	33	0.0327	15.9
1.5	61	0.0199	27	0.0325	10.8
1	66	0.0202	22	0.0316	19.7
0.5	75	0.0087	13	0.0316	5.50



Table S14. Records of the most common insect herbivores observed on *Brassica nigra* plants during three years of field experiments: 2011, 2012 and 2013. Each year 180 plants were monitored. Insect presence was assessed by weekly counts, surveying herbivore community development on individual plants from seedling until seed maturation. An asterisk indicates the insect was used in our greenhouse experiments.

Species	Diet breadth	Feeding guild	Life stage during interaction	Total number of individuals	Average number per plant	Median per plant	Prevalence (% of plants)
<i>Brevicoryne brassicae</i> *	Specialist	Phloem feeder	All	867877	2243	351	79.3
<i>Myzus persicae</i> *	Generalist	Phloem feeder	All	10364	22	19	98.2
<i>Plutella xylostella</i> *	Specialist	Chewer	Larvae	5155	11	9	98.4
Leaf mining flies (<i>Acromyza</i> and <i>Scaptomyza</i>).	Specialist	Chewer	Larvae	2050	9	8	45.1
<i>Phyllotreta undulata</i>	Specialist	Chewer	Adult	1768	6	3	63.9
<i>Phaedon cochleariae</i>	Specialist	Chewer	All	1580	15	6	21.1
<i>Pieris rapae</i> *	Specialist	Chewer	Larvae	1437	4	3	74.8
<i>Lygus</i> spp.	Specialist	Sap feeder	All	1406	3	3	83.8
<i>Phyllotreta atra</i>	Specialist	Chewer	Adult	992	3	2	59.8
<i>Aphis fabae</i>	Generalist	Phloem feeder	All	814	8	2	19.7
<i>Mamestra brassicae</i> *	Generalist	Chewer	Larvae	391	2	1	40.2
<i>Lipaphis erysimi</i> *	Specialist	Phloem feeder	All	327	3	2	22.1
<i>Pieris brassicae</i> *	Specialist	Chewer	Larvae	251	23	1	2.3



Table S14. Continued.

Species	Diet breadth	Feeding guild	Life stage during interaction	Total number of individuals	Average number per plant	Median per plant	Prevalence (% of plants)
Cicade spp.	Generalist	Sap feeder	All	203	1	1	29.7
<i>Myzus persicae</i> sub. <i>nicotianae</i> *	Generalist	Phloem feeder	All	147	3	1	11.1
Weevil spp.	Specialist	Chewer	All	124	1	1	19.5
<i>Evergstris forficalis</i>	Specialist	Chewer	Larvae	102	2	1	9.6
<i>Athalia rosae</i> *	Specialist	Chewer	Larvae	78	1	1	11.7
<i>Autographa gamma</i> *	Generalist	Chewer	Larvae	75	3	2	5.9
Looper caterpillar spp.	Generalist	Chewer	Larvae	41	1	1	6.8
<i>Aleyrodes proletella</i>	Specialist	Sap feeder	All	30	2	1	4.1
<i>Murgantia histrionica</i>	Specialist	Sap feeder	All	21	1	1	4.3
Alticini spp.	Generalist	Chewer	Adult	2	1	1	0.4
<i>Evergstris extimalis</i>	Specialist	Chewer	Larvae	1	1	1	0.2



SUPPLEMENTARY RESULTS AND DISCUSSION

Plant transcriptional responses to herbivore induction

The relative expression of the JA-marker gene *LOX2* strongly depended on the inducing herbivore species (Table S3 and S4). Treatment effects ranged from almost no effect up to an 8-fold increase in transcription of *LOX2* compared to expression in non-treated plants (Extended Data Figure 2A). We observed an increase in relative expression of *LOX2* over time, which was included as an additive effect in the optimal model (Table S3). When relating relative expression of *LOX2* to the feeding behaviour of the herbivore, we found that it depended on the interaction between feeding guild and host specialization of the herbivore with an additive effect of the time after the start of herbivory (Extended Data Figure 2B). At 24 hours since start of herbivory, specialist leaf chewers induced a higher expression of *LOX2* compared to generalist leaf chewers or phloem feeders (Tukey HSD; $P = 0.007$; $P = 0.003$ respectively). At 96 hours after herbivory, specialist leaf chewers induced a stronger *LOX2* expression level than specialist phloem feeders (Tukey HSD; $P = 0.042$), while marginally insignificant when compared to generalist phloem feeders (Tukey HSD; $P = 0.058$). Overall, our findings show that chewing herbivores induce a stronger response of this JA marker gene compared to phloem feeders.

We found no herbivore-specific induction of the SA-marker gene *PR1* (Extended Data Figure 2C; Table S5). Expression of *PR1* could best be explained by an interaction between the herbivore's feeding guild and the time after herbivory, and an additive effect of the herbivore's host specialization. It should however be noted that this model was not considered significantly better than the null model (Table S6; $AIC_{Full} = 511$; $AIC_{Optimal} = 506$; $AIC_{Null} = 508$), indicating that effects are likely to be small. Indeed, post hoc analysis of the optimal model showed only a significantly higher expression of *PR1* at 96 hours in generalist or specialist phloem feeder treatments compared to treatments involving generalist chewers (Tukey HSD; $P = 0.050$; $P = 0.038$ respectively) (Extended Data Figure 2D). Hence, this analysis suggests that phloem feeders induce higher transcription of *PR1* in *B. nigra* compared to leaf chewers, although differences in the induced changes in



expression levels are minor. Overall, the results confirm that leaf chewers induce stronger plant responses characterized by a gene that regulates the JA signal transduction pathway, whereas plant responses to aphids induced stronger plant responses characterized by a gene that is downstream on the SA signal transduction pathway. For chewing herbivores, induction of a JA-related gene *LOX2* was stronger in response to attack by specialists compared to attack by generalists.

Herbivore performance

Performance of the receiver species feeding on plants that were previously induced by one of the ten herbivore species (Table S1) strongly depended on the interaction between the identity of the inducing and the receiving herbivore species (Table S7). This suggests that the outcome of plant-mediated pairwise interactions was highly species-specific (Fig. 1A, Extended Data Figure 2, Table S8). Furthermore, the lack of consistent effects of inducers on receiver performance was corroborated by finding no relationship between the performance of secondary herbivores and the plants transcriptional response (in terms of *LOX2* and *PR1* expression at 96h) (Extended Data Figure 4). Thus, the induced resistance phenotype of the plant as characterized by specific marker genes had little predictive value for the ecological outcome of pairwise interactions, which is mainly determined by the identity of both inducing and receiving herbivores. In *Brassica nigra*, herbivore species may specifically induce changes in primary and secondary metabolites as well as morphological traits such as trichomes. Root herbivores, leaf chewing herbivores and phloem feeders have been found to induce an increase in glucosinolates (van Dam *et al.*, 2006; Ponzio, *et al.*, 2017; Papazian *et al.*, 2019; Hopkins *et al.*, 2009), the main class of secondary metabolites involved in herbivore resistance of Brassicaceae (Hopkins *et al.*, 2009). Phloem feeders and leaf chewers have been found to differ in their effect on primary metabolite profiles in leaves, such as sugar pools (Ponzio, *et al.*, 2017; Papazian *et al.*, 2019). Specialist and generalist herbivores may experience reduced performance on herbivore-induced *B. nigra* plants and the impact of specific defence traits may be herbivore species specific, with stronger negative effects of glucosinolates on generalists than on specialists (Hopkins *et al.*, 2009; Traw *et al.*, 2006).



When testing the effects of the functional groups (host specialization and feeding guild) of both the inducing and receiving herbivore on performance of the secondary herbivore, we found that species-specific effects within feeding guild and host specialization of the receiving herbivore were driving our analysis (Table S9). This confirms that species identity of the secondary herbivore is more important in explaining the outcome of plant-mediated interactions among herbivores than the identity of the inducing herbivore. To allow a more general interpretation of our data we proceeded by exploring a model excluding the random species intercepts driving our previous analysis. This new model retained an interaction between feeding guild of the inducer and host specialization of the receiving herbivore that best explained our results (Fig. 1B, Table S10). Generalist receivers performed better on plants induced by chewing herbivores compared to specialist receivers that fed on plants induced by chewers or by phloem feeders (Tukey HSD; $P < 0.001$ for both comparisons). In addition, generalist receivers performed better on plants induced by phloem feeders compared to specialist herbivores that fed on plants induced by chewing herbivores (Tukey HSD; $P = 0.048$). Overall, we found that generalist chewing herbivores performed better on induced plants compared to their performance on non-treated plants, while specialized chewing herbivores performed better on non-treated plants compared to their performance on induced plants. The feeding guild of the inducing herbivore further amplified differences in the outcome of interactions. For generalist herbivores feeding on induced plants, performance was better on plants induced by chewers than by aphids. It is important to note that performance of generalist chewing herbivores was overall poor on both non-treated and induced plants (Extended Data Figure 3). The small absolute differences in performance between undamaged and induced plants as well as the general high mortality of generalist herbivores suggest that these herbivores are strongly negatively affected by constitutive levels of resistance in *B. nigra* (Hopkins *et al.*, 2009).



Relation to field observations

We found that 53 out of the 90 pairwise interactions we tested in our greenhouse experiment were observed on less than 5% of the plants in the field experiments, and 5 out of 90 interactions were not observed in the field over a three-year period (Fig. 2D, Table S8). Our data show that commonly observed interactions where the second herbivore is a plant specialist are associated with decreasing performance of the receiver relative to its performance on non-treated plants (Fig. 2, Table S12). These common interactions were largely a result of the high prevalence of specialist herbivores (Table S11). The role the prevalence of the receiver had in predicting the outcome of the interactions depended on the specialisation of the receiver and its diet breadth. We found that commonality of the inducer had a small but significant effect on performance of the secondary herbivore depending on the feeding guild of the receiver. Interestingly, this pattern is opposite when the receiver is a generalist. This pattern was robust for a large range of threshold values of what was defined as a common and uncommon interaction (Table S12). Variability in performance was generally lower for interactions we classified as common compared to interactions we classified as uncommon. However, this finding was more sensitive to the choice of the threshold compared to the previous analysis (Table S13). Lower variation in common pairwise-interactions compared to uncommon interactions suggests that uncommon interactions are more unpredictable in terms of receiver performance. These interactions typically consisted of rare secondary herbivores.

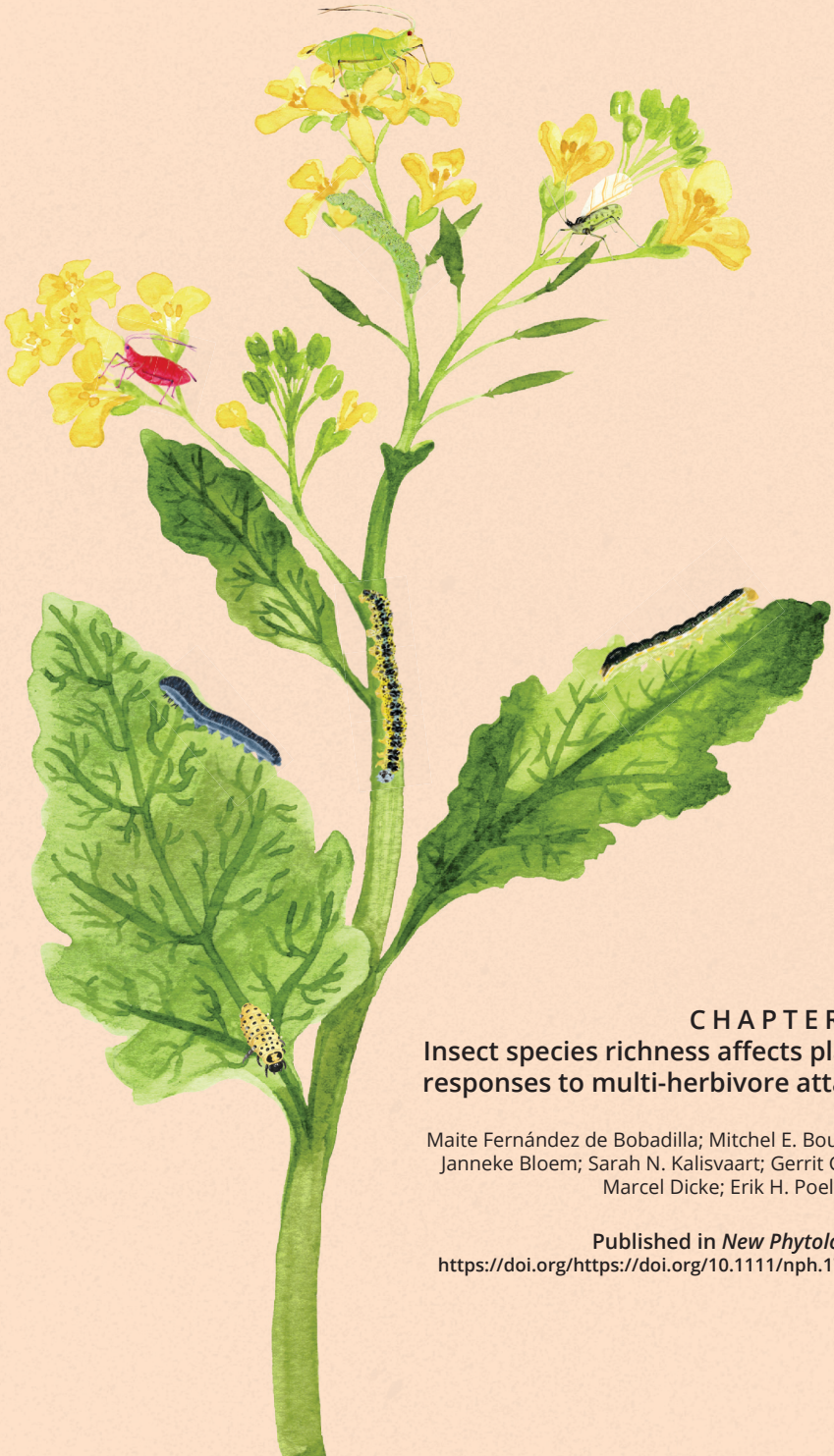
We observed 24 herbivore species in our field experiment (Table S14), realizing 453 out of 552 possible pairwise interactions (Fig. 2D). When ranking the observed interactions by the percentage of plants on which they occur (i.e. interaction frequency), we found an exponential decay in prevalence of the interactions. This indicates that a relatively small set of observed interactions occurred on a large percentage of plants while a large proportion of the interactions could be considered uncommon. On a scale from common to uncommon, the pairwise interactions tested in our greenhouse setup represent an



evenly distributed subset of the 453 interactions observed under field conditions (Fig. 2D), with the exception of five interactions that we did not observe in our field experiment. The interactions tested in our greenhouse experiment that were most common under field conditions involved a subset of only four herbivore species: *Plutella xylostella*, *Myzus persicae*, *Brevicoryne brassicae*, and *Pieris rapae* (11 interactions occurring on more than 25% of the plants in our field experiments). These herbivores are characterized by both high prevalence (i.e. % of plants on which they occur) and abundance in our field experiments (Table S13). The herbivore community developing in our field experiment was increasingly specialist-dominated towards the end of the plants lifetime (Fig. 2C). For chewing herbivores, we found that specialists dominate the community from early stages of community development onward. For phloem feeding herbivores, we observed a generalist-dominated herbivore community early in the season that develops to a specialist-dominated community at the end of the season.

We conclude that plant induced responses to herbivory in *B. nigra* are adaptive in the context of risk of attack by specialist herbivores later in the season. In addition to food plant specialisation, other traits of herbivores may also impact the evolution of induced defence (Tiffin *et al.*, 2006). Mobility of herbivores has been identified to promote selection on induced plant defences, whereas constitutive defences are effective against immobile herbivores that for longer time during a plant's lifetime reside on plants. Induced defences for less mobile herbivores are only favoured when the specific induced traits are highly effective defences against the specific herbivore and the herbivore feeds for a relatively short time on the plant. Induced defences may also be effective in initiating herbivore movement away from plants, limiting the duration of feeding (Rubin *et al.*, 2015).





CHAPTER 4
Insect species richness affects plant responses to multi-herbivore attack

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ABSTRACT

Plants are often attacked by multiple insect herbivores. How plants deal with an increasing richness of attackers from a single or multiple feeding guilds is poorly understood.

We subjected black mustard (*Brassica nigra*) plants to 51 treatments representing attack by an increasing species richness (1, 2 or 4 species) of either phloem feeders, leaf chewers, or a mix of both feeding guilds when keeping total density of attackers constant and studied how this affects plant resistance to subsequent attack by caterpillars of the diamondback moth (*Plutella xylostella*).

Increased richness in phloem-feeding attackers compromised resistance to *P. xylostella*. In contrast, leaf chewers induced a stronger resistance to subsequent attack by caterpillars of *P. xylostella* while species richness did not play a significant role for chewing herbivore induced responses. Attack by a mix of herbivores from different feeding guilds resulted in plant resistance similar to resistance levels of plants that were not previously exposed to herbivory.

We conclude that *B. nigra* plants channel their defence responses stronger towards a feeding-guild specific response when under multi-species attack by herbivores of the same feeding guild, but integrate responses when simultaneously confronted with a mix of herbivores from different feeding guilds.



INTRODUCTION

Plants interact with a community of organisms, from which insects are among the most prominent members. Some of these interactions benefit plant fitness, such as interactions with pollinators or plant growth-promoting microorganisms (Berg, 2009; Pineda *et al.*, 2010; Giron *et al.*, 2018). However, many plant interactions negatively impact plant fitness because they involve organisms that consume plant tissues, such as insect herbivores. In natural ecosystems, plants are commonly attacked by multiple insect herbivores and are under selection to optimise their defences against their community of attackers to maximise their fitness (Lankau & Strauss, 2008; Wise & Rausher, 2013; Poelman & Kessler, 2016). Because the production and maintenance of plant defences is energetically costly, most defence mechanisms are inducible (i.e. only activated upon herbivore feeding) to save the costs of defence in the absence of herbivores (Karban, 2019). To deal with a suite of attackers that may all require different defensive traits, plants have evolved mechanisms to recognize the specific attacker by its feeding guild (e.g. leaf chewer or phloem feeder), feeding pattern, feeding position, or elicitors in its saliva, and tailor the induced defence phenotype to the specific attacking herbivore (Acevedo *et al.*, 2015; Züst & Agrawal, 2016). In this context, plants use a network of phytohormones to regulate responses to specific herbivores with Jasmonic Acid (JA) signalling being dominantly involved in resistance to leaf chewing herbivores and Salicylic Acid (SA) to phloem feeding herbivores (Walling 2000; Pieterse *et al.* 2012; Erb & Reymond, 2019).

When plants are attacked by multiple herbivores, inhibition or potentiation between defence pathways may allow plants to fine tune defence responses to deal with a suite of herbivores (Li *et al.*, 2018; Erb & Reymond, 2019). However, situations where crosstalk between pathways has an apparent negative effect for the plant may also occur (Koornneef & Pieterse, 2008; Thaler *et al.*, 2012; Moreira *et al.*, 2018). Phytohormonal crosstalk such as between SA and JA regulatory pathways may impair plant responses to one insect when the plant has already directed its resistance response towards a previous attacker of a different feeding guild (Pieterse *et al.*, 2012; Soler *et al.*, 2012; Moreira *et al.*, 2018). To optimize



resistance strategies to attack by a suite of herbivores, plants may have to reduce the costs of compromises in resistance to future attack when dealing with the current attacker. Thus, plants may tailor plasticity in defence to arrival patterns of herbivores in the often species rich antagonist communities they are exposed to (Mertens *et al.* 2021). Yet, little research has addressed plant-herbivore interactions beyond dual attack. The few studies that included a third herbivore have identified that the order of attack by two herbivores and which species are involved in the interaction affect plant resistance to subsequent attack by a third herbivore (Mathur *et al.*, 2013; Stam *et al.*, 2014; Stam *et al.*, 2017; Stam *et al.*, 2019). Many plant species are attacked by an insect community that comprises more than three species, but how plants deal with their common situation of multi-herbivore attack is poorly understood. A myriad of factors may be relevant for plant responses to multi-herbivore attack. This includes the variation in herbivore traits such as feeding guild or food plant specialisation, the order of arrival of herbivores and timing of herbivory during plant ontogeny. At the same time, diversity *per se* or components of diversity may affect species interactions (Loreau & Hector, 2001; Soliveres *et al.*, 2016). Before disentangling the significance of each of these components, it should be established whether plants do respond differently to attack by a larger number of herbivore species than to a few and whether it matters that these species are more or less similar in traits.

In this study, we tested if species richness of previous attackers affects a plant's capability to respond to a subsequent attacker of the same or of a different feeding guild. We hypothesized that an increasing richness of initial attackers leads to stronger feeding-guild-specific plant responses when all herbivores are of the same feeding guild than when plants are attacked by a mix of herbivores from different feeding guilds. The stronger response to a higher richness of attackers of the same feeding guild is expected to lead to increased resistance to subsequent attack by a herbivore of the same feeding guild, but to increased susceptibility to attack by a herbivore of a different feeding guild. To test these hypotheses, we subjected black mustard (*Brassica nigra*) plants to attack by an increasing richness (1, 2 or 4 species) of either phloem-feeding, leaf-chewing



herbivores, or a mix of both. Over twenty different herbivore species may be found on *B. nigra* plants, but over their lifetime individual plants are on average colonised by eight different herbivore species out of this herbivore species pool (Mertens *et al.*, unpublished). We selected eight of the most prevalent herbivore species equally distributed across the leaf chewer and phloem feeder guilds and studied how plant responses to these attackers affect resistance to subsequent attack by caterpillars of the diamondback moth (*Plutella xylostella*). Caterpillars of *P. xylostella* typically attack *B. nigra* later in the season when plants have been previously attacked by other herbivores and virtually all plants in a stand are attacked by *P. xylostella* (Mertens *et al.*, unpublished). In addition, we quantified how combinations of these herbivores affect the expression of marker genes of the JA- and SA- phytohormonal pathways to characterise induced plant resistance to multi-herbivore attack. By identifying how plants deal with increased species richness of attack, we signal the importance of placing plant defence plasticity in the context of plant-insect community interactions.









MATERIALS AND METHODS

Plants and insects

Three-to-four-week-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, The Netherlands (51° 57' 32" N, 5° 40' 23" E). The plants and the insects were cultured in a greenhouse at 22 ± 2 °C, 60-70 % RH and 16:8 h L:D photo regime. Eight insect species were used as primary herbivores or inducers of plant defence (Table 1). We used first-instar larvae of herbivores of a leaf-chewing feeding guild: the cabbage moth, *Mamestra brassicae* (*Mb*) (Lepidoptera: Noctuidae); the large cabbage white, *Pieris brassicae* (*Pb*) (Lepidoptera: Pieridae); the turnip sawfly, *Athalia rosae* (*Ar*) (Hymenoptera: Tenthredinidae); and the mustard leaf beetle, *Phaedon cochleariae* (*Pc*) (Coleoptera: Chrysomelidae). For members of the phloem-feeding guild we used the cabbage aphid, *Brevicoryne brassicae* (*Bb*); the green peach aphid, *Myzus persicae* (*Mp*); the tobacco aphid, *Myzus persicae* sub. *nicotianae* (*Mpn*); and the mustard aphid, *Lipaphis erysimi* (*Le*) (all Hemiptera: Aphididae) (Table 1).



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

Species (Abbreviation)	Picture	Order	Feeding guild
<i>Athalia rosae</i> (Ar)		Hymenoptera	Leaf chewer
<i>Mamestra brassicae</i> (Mb)		Lepidoptera	Leaf chewer
<i>Phaedon cochleariae</i> (Pc)		Coleoptera	Leaf chewer
<i>Pieris brassicae</i> (Pb)		Lepidoptera	Leaf chewer
<i>Brevicoryne brassicae</i> (Bb)		Hemiptera	Phloem feeder
<i>Lipaphis erysimi</i> (Le)		Hemiptera	Phloem feeder
<i>Myzus persicae</i> (Mp)		Hemiptera	Phloem feeder
<i>Myzus persicae</i> sub. <i>nicotianae</i> (Mpn)		Hemiptera	Phloem feeder

As a subsequent herbivore (or receiver), second-instar larvae of the diamondback moth *P. xylostella* (Lepidoptera: Plutellidae) were used. Larvae of this insect are specialists on brassicaceous plants and feed on foliar tissue, buds and flowers. *Mamestra brassicae*, *P. brassicae*, *B. brassicae* and *P. xylostella* were reared on Brussels sprouts plants (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus). *Myzus persicae*, *M. persicae* sub. *nicotianae*, *L. erysimi*, *A. rosae* and *P. cochleariae* were reared on radish (*Raphanus sativus*). All insects were obtained from the stock rearing of the Laboratory of Entomology, Wageningen University and were maintained at $22 \pm 2^\circ\text{C}$, 60–70 % RH and 16:8 h L:D photo regime.

Induced resistance to *P. xylostella* after multi-herbivore attack

Species richness effect within feeding guilds

We first assessed how an increase in richness of attackers affects induced plant resistance to subsequent herbivory by *P. xylostella* caterpillars feeding on induced plants. Due to the large scale of our study and its associated constraints in terms of greenhouse space as well as numbers of caterpillars that can be weighed on a single day, we conducted separate experiments for leaf-chewers



(CHEW), phloem-feeders (PHLO) and a mix of species of these feeding guilds (MIX). We thus focussed on quantifying the effect of species richness within each feeding guild.

We assessed plant responses to different levels of herbivore richness by infesting each plant with either one (leaf-chewer: CHEW-1; phloem-feeder: PHLO-1), two (CHEW-2; PHLO-2; MIX-2) or four (CHEW-4; PHLO-4; MIX-4) herbivore species while keeping total herbivore numbers constant. We excluded richness of three species, because of the imbalance that these treatments would have in number of leaf-chewer and phloem-feeder species. Each experiment included control plants that did not receive any inducing herbivore but were treated in a similar way as plants receiving herbivores (CON-O). The total density used was four leaf chewers and eight phloem feeders for the leaf-chewer and phloem-feeder experiment respectively, and two leaf chewers plus four phloem feeders for the mixed richness experiment. We chose the number of species and the total density of inducers used based on field observations of insect communities of *B. nigra* plants (Poelman *et al.*, 2009; Mertens *et al.*, unpublished). We allowed population growth of the initial infestation of the eight adult phloem feeders. For the mixed richness experiment, MIX-2 resulted in 16 insect combinations of a single phloem-feeder and leaf-chewer, that were all tested. For MIX-4 a selection of 12 of the 36 possible combinations of two phloem-feeders and two leaf-chewers were tested, selected to have the biggest differences between species combinations, while each herbivore is present the same number of times (6 times) (Table S1). Thus, while testing the effect of leaf-chewer richness we compared the performance of *P. xylostella* caterpillars on: 1) control plants (CON-0), 2) plants induced by four larvae of a single chewer species (CHEW-1, for each of the four chewer species), 3) plants induced by two larvae of two chewer species (CHEW-2, for each of the six species combinations), 4) plants induced by four larvae, one of each of the four chewer species (CHEW-4). For the phloem-feeder richness experiment the setup was similar to the leaf-chewer richness, with the only difference that the total number of inducer individuals was eight. For the mixed richness, we compared



the performance of *P. xylostella* caterpillars on: 1) control plants (CON-0), 2) plants induced by two larvae of one chewer species plus four phloem feeders of one species (MIX-2, for each of the sixteen species combinations), 3) plants induced by a single larva of two chewer species plus four phloem feeders (two of each species) (MIX-4, for each of the twelve species combinations selected).

Each experiment was divided into two temporal blocks. We prepared a total of ten plant replicates (five per block) per insect combination for the leaf-chewer and phloem-feeder richness experiment, and eight plant replicates (four per block) per insect combination for the mixed richness experiment (Table 2). To prevent dehydration and cross-contamination between treatments, 4-5 plants receiving the same herbivore combination were placed jointly in trays inundated with water. All treatments with unique species (combinations) replicating the species richness levels were randomized across the greenhouse. Insects were placed on the youngest fully expanded leaf of the plant and could freely feed from the plant for one week (Fig. 1). Movement of herbivores between plants was prevented by water surrounding the pots in the inundated trays. The inducing herbivores were removed with a brush, after seven days of feeding to exclude direct effects of inducing herbivores on the receiver herbivore. One

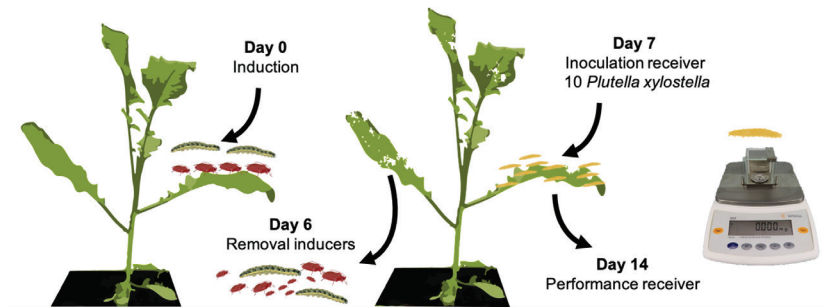





Figure 1. Experimental setup used to measure the performance of *P. xylostella* caterpillars on plants previously attacked by different species richness of phloem feeders, chewers or a mix of both (51 insect combinations).



Table 2.

Group	Species richness	Treatment	Performance			Gene expression	
			Plant replicates	Trays	Replicates group	Biological replicates	Replicates group
 Leaf chewers	0	Ctrl	10	2	10	5	5
	1	<i>Mb</i>	10	2	40	5	20
		<i>Ar</i>	10	2		5	
		<i>Pb</i>	10	2		5	
		<i>Pc</i>	10	2		5	
		<i>Mb-Ar-</i>	10	2		5	
	2	<i>Mb-Pb</i>	10	2	60	5	30
		<i>Mb-Pc</i>	10	2		5	
		<i>Ar-Pb</i>	10	2		5	
		<i>Ar-Pc</i>	10	2		5	
		<i>Pb-Pc</i>	10	2		5	
	4	<i>Mb-Ar-Pb-Pc</i>	60	12	60	20	20
 Phloem feeders	1	<i>Bb</i>	10	2	40	5	20
		<i>Mp</i>	10	2		5	
		<i>Mpn</i>	10	2		5	
		<i>Le</i>	10	2		5	
		<i>Bb-Mp</i>	10	2		5	
	2	<i>Bb-Mpn</i>	10	2	60	5	30
		<i>Bb-Le</i>	10	2		5	
		<i>Mp-Mpn</i>	10	2		5	
		<i>Mp-Le</i>	10	2		5	
		<i>Mpn-Le</i>	10	2		5	
	4	<i>Bb-Mp-Mpn-Le</i>	60	12	60	20	20
	 +	2	<i>Bb-Mb</i>	8	2	128	2
<i>Bb-Ar</i>			8	2	2		
<i>Bb-Pb</i>			8	2	2		
<i>Bb-Pc</i>			8	2	2		
<i>Mp-Mb</i>			8	2	2		
<i>Mp-Ar</i>			8	2	2		
<i>Mp-Pb</i>			8	2	2		
<i>Mp-Pc</i>			8	2	2		
<i>Mpn-Mb</i>			8	2	2		
<i>Mpn-Ar</i>			8	2	2		
<i>Mpn-Pb</i>			8	2	2		
<i>Mpn-Pc</i>			8	2	2		
<i>Le-Mb</i>			8	2	2		
<i>Le-Ar</i>			8	2	2		
<i>Le-Pb</i>			8	2	2		
<i>Le-Pc</i>		8	2	2			
4		<i>Bb-Mp-Mb-Ar</i>	8	2	96	2	24
		<i>Bb-Mp-Pb-Pc</i>	8	2		2	
		<i>Bb-Mpn-Mb-Pc</i>	8	2		2	
		<i>Bb-Mpn-Ar-Pb</i>	8	2		2	
		<i>Bb-Le-Mb-Pb</i>	8	2		2	
		<i>Bb-Le-Ar-Pc</i>	8	2		2	
		<i>Mp-Mpn-Mb-Pb</i>	8	2		2	
		<i>Mp-Mpn-Ar-Pc</i>	8	2		2	
		<i>Mp-Le-Mb-Pc</i>	8	2		2	
		<i>Mp-Le-Ar-Pb</i>	8	2		2	
		<i>Mpn-Le-Mb-Ar</i>	8	2		2	
		<i>Mpn-Le-Pb-Pc</i>	8	2		2	



day later, each plant was infested with 10 second instar *P. xylostella* larvae, acting as subsequent herbivore, or receiver. The mass of *P. xylostella* caterpillars was measured after six days of feeding on the control and induced plants as a proxy of plant resistance. This was done by recapturing the *P. xylostella* larvae and weighing each individual on a Sartorius® - CP2P - Analytical Balance (accuracy 0.001 mg).

Comparing direction of effects by feeding guilds

After establishing in separate experiments for feeding guild the effect of species richness within feeding guild on the performance of *P. xylostella* and in which direction each feeding guild affected *P. xylostella* performance, we performed an additional experiment to directly compare the effect of feeding guild on *P. xylostella* performance. We selected the most extreme treatments of each main experiment: CON-0 (control), phloem-feeder (PHLO-4), leaf-chewer (CHEW-4) and mixed (MIX-4) and prepared 24 plant replicates per treatment (Table S2). We randomized individual plants over the greenhouse and followed the same protocol of inducing the plants, removing the herbivores used for induction and assessing the performance of *P. xylostella* caterpillars on these plants. All performance experiments were performed in the same greenhouse compartment (December 2017 to July 2018) (19°C, 60-70 % RH and 16:8 h L:D photo regime).

Table 2. Overview of treatments and replicates used for performance of *Plutella xylostella* experiment (approach 1) and for the experiment where we measured expression of defence related genes on *Brassica nigra* leaves. We show replicates per treatment and per species richness within each group (leaf chewer, phloem feeder, mix). *P. xylostella* performance was measured in three separate experiments (leaf chewer, aphid, mix), each of them divided in two blocks over time. Each of these blocks contained five control plants (species richness 0, no insects). The replicates shown are the total of replicates for the two blocks together. Each replicate consisted of one *B. nigra* plant where we inoculated ten *P. xylostella*. The gene expression analysis was done in a single experiment. Here, each biological replicate consisted of a pool of leaf disks from three plants. We prepared a set of biological replicates that were sampled 48h after induction, and another set that was sampled at 96h after induction. Each plant was sampled only once. Leaf chewsers: Mb= *Mamestra brassicae*, Ar= *Athalia rosae*, Pb= *Pieris brassicae*, Pc= *Phaedon cochleariae*. Aphids: Bb= *Brevicoryne brassicae*, Mp= *Myzus persicae*, Mpn= *Myzus persicae* sub. *nicotianae*, Le= *Lipaphis erysimi*.



Analysis of B. nigra gene expression

We characterised *B. nigra* physiological responses to attack by the herbivore combinations in a single separate experiment (same treatments used for measuring performance, Table 2). The experiment was conducted in a different greenhouse compartment than the performance tests, but with similar climate conditions (July-August 2018) (19°C, 60-70 % RH and 16:8 h L:D photo regime). To characterise plant responses, we selected homologues of genes that are known to regulate JA- and SA-plant responses to insect-herbivore attack. We analysed expression levels of the JA-biosynthesis gene *LIPXYGENASE 2 (LOX2)*, the JA-responsive gene *VEGETATIVE STORAGE PROTEIN2 (VSP2)*, the SA-biosynthesis gene *ISOCHORISMATE SYNTHASE (ICS)*, the SA-responsive gene *PATHOGENESIS-RELATED PROTEIN 1 (PR1)* and the crosstalk gene *WRKY70*. Biosynthesis of the lipid-derived phytohormone JA starts when injury of plant cells releases α -linolenic acid from the plastid membranes (Wasternack & Song, 2017). A key regulator of the first steps in JA biosynthesis is the enzyme lipoxygenase (LOX) which activates expression of JA-biosynthetic genes, such as *LOX2* (Bell *et al.*, 1995). The bioactive form of JA (JA-Ile) is synthesized after a few enzymatic steps and transported to the nucleus. JA-Ile triggers the degradation of the *JASMONATE-ZIM DOMAIN (JAZ)*, a repressor of JA biosynthesis. JAZ degradation releases repression of MYC transcription factors, resulting in expression of JA-responsive genes, such as *VSP2* (Erb & Reymond, 2019). SA can be synthesized via the *PHENYLALANINE AMMONIOLYASE (PAL)* and the *ISOCHORISMATE SYNTHASE (ICS)* enzymatic pathways (Pieterse *et al.*, 2012). Signalling downstream of SA biosynthesis is regulated by transcription factors that activate expression of pathogenesis-related defence genes (*PR* genes) (Pieterse *et al.*, 2012). Additionally, WRKY transcription factors play a key role in SA signalling, as they activate or repress SA responses.

We focussed on characterising how plants respond to attack when herbivores are coming from the same feeding guild (phloem-feeders or leaf-chewers) or from multiple feeding guilds. Within feeding guild, we then



addressed whether individual herbivores, number of herbivore species and specific combinations differ in induced plant responses. Gene expression in response to herbivory was assessed at 48 and 96 hours after induction, using a different set of plants per time point so that plants were only sampled once. Our earlier experiments with *B. nigra* identified that with the two time points the expression patterns of the selected genes can be well characterised (Mertens *et al.*, unpublished). We prepared five biological replicates per treatment for control (CON-0), leaf-chewer (CHEW-1; for each of the four leaf-chewer species) and phloem-feeder (PHLO-1; for each of the four phloem-feeder species) and five replicates for each CHEW-2 and PHLO-2 species combination (six species combinations for leaf chewers and phloem feeders) (Table 2). We prepared 20 biological replicates for treatments with four herbivore species CHEW-4 and PHLO-4 to balance the number of replicates across species richness treatments. MIX-2 (16 species combinations) and MIX-4 (12 species combinations) had two biological replicates per species combination, which resulted in 32 biological replicates for MIX-2 and 24 for MIX-4, respectively. Each biological replicate contained a leaf disc ($\phi = 2$ cm) that was taken with a sterilized puncher from three plants with the same treatment, sampling each plant only once. Leaf samples were placed in an Eppendorf tube, immediately flash frozen in liquid nitrogen and stored at -80 °C until further analysis (Fig. 1). Induction of plants with the herbivore treatments was performed on the youngest fully-expanded leaf. Directly after induction, the induced leaf was enclosed in a mesh bag to confine herbivores to a single leaf. Control plants received a mesh bag without insects. Biological replicates represented by the group of three plants that would be combined in a single sample were randomly placed in the greenhouse.

RNA extraction and PCR protocol

The frozen samples were ground to a fine powder with a sterile pestle. This was followed by RNA extraction using the RNeasy 96 Kit (Qiagen) with an adjusted protocol for plants. 450 μ L of RTL buffer were added to each Eppendorf tube (which contained approximately 50 mg of frozen, ground leaf sample). Samples were incubated at 56 °C for 1-3 minutes and were centrifuged



at 10,000 Relative Centrifugal Force (RCF), until a tight pellet was formed (8-10 minutes). The supernatant was transferred to new Eppendorf tubes and mixed with 0.5 volumes of 96% ethanol. Each sample was transferred to a 96-RNeasy plate and that was centrifuged 6 minutes at 3700 RCF. The flow-through was discarded, and each well was treated with 80 μ L of a solution of DNase I in RDN buffer (1:7) which was added to the tube directly onto each membrane and was incubated for 15 minutes at room temperature. To each sample, 800 μ L of RWI buffer were added, and after 5 minutes of incubation the plate was centrifuged for 6 minutes at 3700 G and the flow-through was discarded. Now, 800 μ L of RPE buffer were added to each sample, and the plate was centrifuged for 6 minutes at 3700 RCF, the flow-through was discarded and the plate was centrifuged for 10 minutes at 3700 RCF. The plate was placed on a rack of collection microtubes, where 45 μ L of RNase free water (Qiagen) were added to each sample and after one-minute incubation, the plate was centrifuged for 6 minutes at 3700 RCF. The RNA concentration was measured using a DS-II FX Spectrophotometer/Fluometer (DeNovix). Samples were diluted and adjusted to an RNA concentration of 66.6 ng/ μ L. From the RNA samples cDNA was synthesized, using the SensiFAST cDNA synthesis kit (Bioline). We quantified the expression levels of the target genes *LOX2*, *VSP2*, *ICS* and *PR1* and of the reference genes *GAPDH* and *SAR1A* by qPCR using the SensiFAST SYBR no-ROX kit (Bioline). We added 5 ng of the cDNA template to the reaction with a total volume of 20 μ L. The reactions were performed using a CFX96 Touch™ Real-Time PCR Detection System (Biorad). All reactions were conducted using two technical replicates and samples were omitted from further analyses if the difference in expression of technical replicates was higher than 0.5 (4% of the samples). Plate setups included negative controls (no template) and inter-run calibrators.

We tested the following reference genes: *ACTIN-2* (*ACT2*), *BETA-TUBULINE* (*B-TUB*), *ELONGATION FACTOR-1* (*EF1*), *PEROXIDASE 4* (*PER4*), *SECRETION ASSOCIATED RAS RELATED GTPASE 1A* (*SAR1A*), *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE*



(*GAPDH*). The last two reference genes were selected because they had highest expression stability regardless of the treatment (primer sequences are presented in Table S3). We used the expression of the reference genes to calculate the relative expression of genes of interest in each sample subjected to the different treatments.

Statistical analysis

Performance data

We first analysed for each of the feeding guilds separately how species richness of inducing herbivores affected plant resistance to the subsequent feeding by *P. xylostella* caterpillars. The performance data (weight of individual *P. xylostella* caterpillars) was cube-root transformed (third level root transformation) to better approach the assumptions of normality and constant variance. We fitted Mixed Linear Models (MLM), using a fixed effect for species richness with the levels of richness 1, 2 and 4 for the model on phloem feeders and the model for leaf-chewers, the levels 2, 4 for the model of the mixed feeding guilds. We included the sub-treatments of each of the unique species or species combinations (11 for phloem feeders, 11 for leaf chewers, and 28 for mix). We present the outcomes of these models with two sets of random effects (Table S4). One set of models that included the random structure for blocks, trays within blocks, plants within trays, and residual error. The second set of models excluded the tray effect from the random structure. Even though in this particular statistical approach the random factor “tray” unexpectedly explained a substantial amount of variation, we purposely removed this factor from the model. The tray corresponded with the variation of sub-treatments of species combinations within the richness levels and likely at least to an extent contains the biological variation caused by unique species combinations. We kept a smaller scale random factor of plant, and we aimed to test the biological effect of the sub-treatments within each main experiment (species included in phloem-feeder, leaf-chewer and mixed treatments) represented by trays with sub-treatments randomly distributed in the greenhouse. Based upon the fitted model, by averaging over the insect combinations that constituted a richness level



within guild, and combining the estimated means linearly, we estimated linear regression lines for species richness within guild, using facilities for user-defined linear combinations of the software. Next, we tested nullity of slopes within guilds to identify whether there was a significant positive or negative correlation between species richness of inducers and performance of *P. xylostella* (slopes excluded performance of caterpillars on undamaged plants). Besides linear trends, also quadratic trends were tested. Since both analyses gave similar results, we present only linear trends in the results section. With the Mixed Model, we then tested for significance of the different richness levels with performance of the caterpillars on undamaged control plants. These analyses were followed by post-hoc analyses to compare individual sub-treatments. Finally, we compared the slopes for the three feeding guilds obtained in the separate experiments among each other to identify whether relationships between species richness and *P. xylostella* performance differed for the feeding guilds. Because this was an indirect comparison combining data obtained at different moments, we had performed an additional experiment that directly compared performance of *P. xylostella* on undamaged control plants, and the most extreme richness of phloem-feeders, leaf-chewers or a mix of feeding guilds. Here individual plants were placed randomly distributed in the greenhouse and a single block was performed. These data were analysed with a Mixed Model for the fixed treatment effect feeding guild (CON-0, PHLO-4, CHEW-4, MIX-4). For the random part, besides the residual variance, only random effects for plants were included.

Statistical analysis for herbivore performance was done in R version 3.6.1 (R Core Team, 2019) and using PROC MIXED of SAS (version 6.4). For all hypothesis tests we used 0.05 as level of significance.

Gene expression

Gene-expression data was imported to qBase+ 3.1 (Biogazelle) to calculate the relative gene expression level between different samples for a given target gene (*LOX2*, *VSP2*, *ICS*, *PR1*, *WRKY70*) corrected by the expression



value of the reference genes (*GAPDH*, *SAR1A*). Data was corrected for differences between runs using inter-run calibrators and data was Log₁₀ transformed to meet the assumptions of the model. For each of the five genes and separately per time point, we ran General Linear Models (GLM) with Post-Hoc test (LSD) in R version 3.6.1 (R Core Team, 2019). We first tested whether the nine different treatment groups based on the combination of feeding guild and species richness differed in expression levels. These analyses identified that expression patterns were strongly determined by feeding guild. We then deepened the analysis by testing in separate models per feeding guild whether the species richness levels significantly affected gene expression, and used post-hoc analyses to compare individual richness levels within feeding guild as well as individual sub-treatments represented by the unique herbivore combinations within feeding guild.

RESULTS

Species richness affects induced plant resistance

Plants attacked by a higher species richness of phloem feeders became more susceptible to caterpillars of the subsequent attacker *P. xylostella* as indicated by an increased caterpillar performance on plants induced by larger number of aphid species (Significant positive slope of species richness 1-2-4, with tray: $P = 0.095$; without tray $P < 0.01$; Fig. 2a). A richness of four aphid species resulted in significant susceptibility to *P. xylostella* compared to its performance on undamaged control plants and susceptibility increased when four species induced plants compared to one or two aphid species (Mixed Model; without tray $F_{3,438} = 4.66$; $P = 0.0032$) (Fig 3a). Individual attack by each of the four phloem-feeding species only slightly enhanced performance of *P. xylostella* to levels not significantly different from performance on undamaged plants (Fig. 3b). However, the specific combination of initial attack by the aphid species *M. persicae* sub. *nicotianae* – *M. persicae*, *M. persicae* sub. *nicotianae* – *L. erysimi* or *L. erysimi* – *B. brassicae* significantly enhanced performance of *P. xylostella* caterpillars (Fig. 3b).



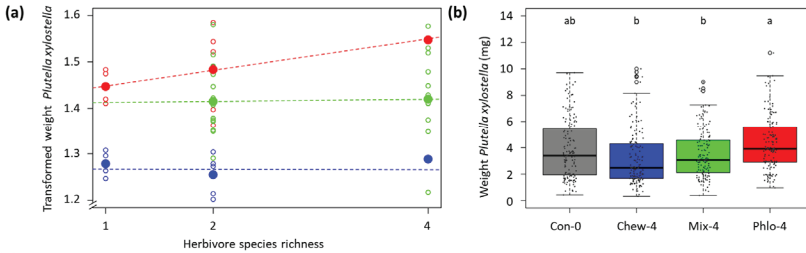


Figure 2. (a) Regression line of the transformed performance of *Plutella xylostella* on *Brassica nigra* plants. Performance of caterpillars in different experimental blocks is presented in weight corrected to the average weight of *P. xylostella* on untreated control plants across all blocks. Plants were treated with an increasing richness (1, 2 or 4 species) of phloem feeders (red), leaf chewers (blue), or a mix of both (green), or were left untreated. The open circles represent averages within each sub-treatment (i.e. insect combination). The phloem-feeder and leaf-chewer experiments contained four sub-treatments for species richness 1, six sub-treatments for species richness 2 and one sub-treatment for species richness 4. The mixed species richness experiment contained sixteen sub-treatments for mixed species richness 2 and twelve sub-treatments for mixed species richness 4. The closed circles represent averages of the sub-treatments within each species richness level. Lighter-coloured circles show treatments that only received 1 herbivore species, middle-dark-coloured circles show treatments that received 2 herbivore species, darker-coloured circles show treatments that received 4 herbivore species. (b) Performance of *P. xylostella* on approach 2: In one experiment, plants were treated with four species of phloem feeders (Phlo-4), leaf chewers (Chew-4), a mix of both (Mix-4) or left untreated (Con-0) ($N = 24$). 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). Letters for panel c can be found in Table S4. Leaf chewers: *Mamestra brassicae* (Mb), *Phaedon cochleariae* (Pc), *Pieris brassicae* (Pb), *Athalia rosae* (Ar). Phloem-feeding aphids *Myzus persicae* sub. *nicotianae* (Mpn), *Lipaphis erysimi* (Le), *Myzus persicae* (Mp), *Brevicoryne brassicae* (Bb).

In contrast, a higher species richness of leaf-chewers did not lead to effects on resistance to *P. xylostella* (No significant slope of species richness 1-2-4; with tray $P = 0.97$; without tray $P = 0.97$; Fig. 2a). Plant induced responses to feeding by one, two or four species of leaf-chewers equally enhanced resistance to *P. xylostella* caterpillars as indicated by decreased performance of these caterpillars compared to their performance on undamaged control plants (Mixed Model; without tray $F_{3,248} = 5.39$; $P = 0.013$) (Fig. 3b). All four leaf-chewing herbivores reduced *P. xylostella* performance similarly when attacking the plant as individual species (Fig. 3a). The combination of the leaf chewers



A. rosae and *P. cochleariae* had the strongest negative effect on the performance of *P. xylostella* caterpillars, but did not differ significantly from other treatments with two leaf-chewer species (CHEW-2) or plants damaged by single chewer species (CHEW-1) or the full set of four species (CHEW-4) (Fig. 3a). Induction of plants by a mix of phloem-feeding and leaf-chewing herbivores with either two or four different species did not affect the performance of *P. xylostella* caterpillars (Slope for richness 2 and 4 not different from 0: with tray $P = 0.81$; without tray $P = 0.81$) (Fig. 2a) and on average the performance of *P. xylostella* on plants induced with a mix of feeding guilds was not different from its performance on undamaged control plants (Mixed Model; without tray $F_{2,485} = 0.03$; $P = 0.97$) (Fig. 3c). Despite these overall neutral effects, plants previously attacked by a specific combination of *M. persicae* sub. *nicotianae* – *A. rosae* or a mix of the four species *M. persicae* sub. *nicotianae* – *L. erysimi* – *M. brassicae* – *P. cochleariae* became less resistant to larvae of *P. xylostella* (Table S5, Fig. 3c). In contrast, plants previously attacked by a mix of the four species *B. brassicae* – *M. persicae* – *P. brassicae* – *A. rosae* became more resistant to larvae of *P. xylostella* (Table S5, Fig. 3c).

In our second independent experiment where individual plants could be randomly distributed in the greenhouse, we confirmed that performance of *P. xylostella* caterpillars differed for plants induced by phloem-feeders, leaf-chewers or a mix of herbivores from different feeding guilds (Mixed Model, $F_{3,77} = 4.37$, $P = 0.0068$) (Fig. 2b). Direct comparison of *P. xylostella* performance on plants induced by species richness of four herbivores supported that phloem-feeders increased performance of *P. xylostella* caterpillars and differed from the reduced performance of caterpillars feeding on leaf-chewer induced plants (Fig. 2b). Caterpillars feeding from plants induced by a mix of feeding guilds had intermediate performance and had significantly lower average mass compared to caterpillars feeding on phloem-feeding induced plants (Fig. 2b). Indirect comparison of the direction of slopes in the first series of experiments further supports that the direction of effect of species richness differs between phloem-feeders and the slope of leaf-chewers or the mixed



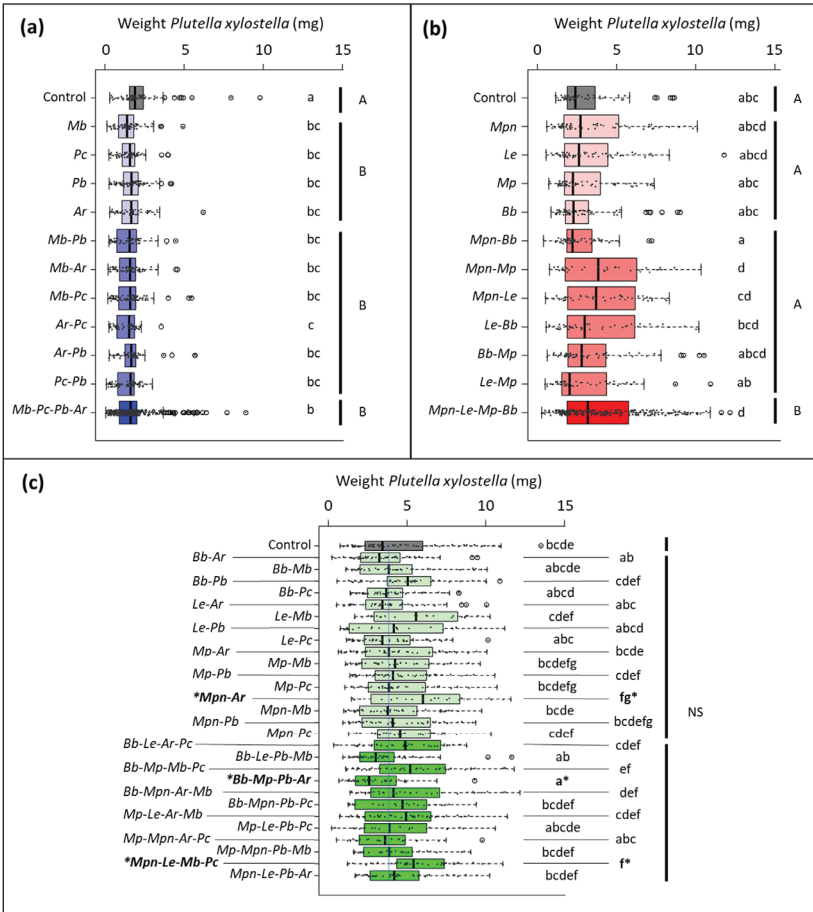


Figure 3. Weight (mg) of *Plutella xylostella* larvae after feeding for six days from *Brassica nigra* plants previously attacked by leaf chewers (blue), phloem feeders (red), a mix of both (green), or from untreated plants (grey, control). Panels (a), (b) and (c) show the performance of *P. xylostella* in approach 1, where we measured performance in three separate experiments. Lighter boxplots show treatments that only received 1 herbivore species, middle-dark boxplots show treatments that received 2 herbivore species, darker boxplots show treatments that received 4 herbivore species. (a) Performance of *P. xylostella* on plants previously attacked by leaf chewers (N = 10). (b) Performance of *P. xylostella* on plants previously attacked by phloem feeders (N = 10) (c) Performance of *P. xylostella* on plants previously attacked by a mix of leaf chewers and phloem feeders



(N= 10 for control, N= 8 for the rest). Combination of inducers that affected *P. xylostella* growth (compared to control, untreated plants) are marked in bold and with an asterisk. 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). Letters for panel c can be found in Table S4. NS= not significant Leaf chewers: *Mamestra brassicae* (*Mb*), *Phaedon cochleariae* (*Pc*), *Pieris brassicae* (*Pb*), *Athalia rosae* (*Ar*). Phloem-feeding aphids *Myzus persicae* sub. *nicotianae* (*Mpn*), *Lipaphis erysimi* (*Le*), *Myzus persicae* (*Mp*), *Brevicoryne brassicae* (*Bb*).

herbivore treatments (PHLO versus CHEW with tray P = 0.22, without tray P < 0.05; PHLO versus MIX with tray P = 0.24, without tray P < 0.05; CHEW versus MIX with tray P = 0.86, without tray P = 0.84).

To summarize, species richness of initial attackers as well as species composition across feeding guilds affects induced plant resistance to subsequent herbivory. Plants attacked by phloem-feeding insects became more vulnerable to larvae of *P. xylostella*, whereas plants attacked by leaf chewers became more resistant to *P. xylostella* caterpillars. Species richness of phloem feeders was more important in affecting *P. xylostella* performance than richness of leaf chewers. For leaf-chewers the presence of a single herbivore species caused similar effects compared to four species of leaf-chewing herbivores. *Plutella xylostella* caterpillars performed equally well on plants induced with a mix of herbivore species from the two feeding guilds regardless of species richness and on plants that had not been exposed to herbivores.

Feeding guild specific induced gene expression

Brassica nigra attacked during 48 and 96h by leaf chewers, or by a mix of leaf chewers and phloem feeders showed induced expression of the JA-biosynthesis and JA-responsive genes (*LOX2* and *VSP2*, respectively) and of the SA-responsive gene (*PR1*) (LM 48h: *LOX2* $F_{8,149} = 20.58$, $P < 0.001$; *VSP2* $F_{8,149} = 24.93$, $P < 0.001$; *PR1* $F_{8,149} = 4.64$, $P < 0.001$, Fig. 4; LM 96h: *LOX2* $F_{8,149} = 43.70$, $P < 0.001$; *VSP2* $F_{8,149} = 18.0$, $P < 0.001$; *PR1* $F_{8,149} = 3.51$, $P < 0.001$, Fig. S1). In contrast, phloem feeders did not induce significant expression of these genes compared to undamaged control plants. None of the herbivore treatments significantly affected expression of the SA-



biosynthesis gene *ICS* and of the crosstalk gene *WRKY70* at either 48 or 96h of herbivore attack (LM, 48h: *ICS* $F_{8,149} = 1.0$, $P = 0.44$; *WRKY70* $F_{8,149} = 1.35$ $P = 0.22$, Fig. 4; LM 96h: *ICS* $F_{8,149} = 1.08$, $P = 0.38$; *WRKY70* $F_{8,149} = 1.12$ $P = 0.30$, Fig. S1). 48 and 96h of feeding by the leaf chewers and by each of the leaf-chewer species combinations induced the JA-biosynthesis gene *LOX2* (Fig. S2 & S3). In addition to the direction of plant responses driven by feeding guild, within feeding guilds species richness affected gene expression only for leaf-chewing herbivores (Figs. S2-S5). For leaf-chewing herbivores, 96h of feeding by a combination of two herbivores enhanced gene expression of *LOX2* ($F = 12.16$, $P < 0.001$) and *VSP2* ($F = 4.54$, $P < 0.01$) compared to single herbivore attack (Fig. S3). Herbivory by four species could not be discriminated in gene expression patterns from those induced by one or two leaf-chewing herbivores (Fig. S3). Individual leaf-chewer species and combinations of specific leaf chewers differed in the magnitude of induced expression of *LOX2* (Fig. S3a). Plants attacked by *P. cochleariae* or by *A. rosae* had stronger *LOX2* induction than plants attacked by *M. brassicae*. Feeding by the combinations of *A. rosae* – *P. cochleariae*, *A. rosae* – *P. brassicae* or *P. brassicae* – *P. cochleariae* induced the highest expression levels of *LOX2* (Fig. S3a). Similarly, most of the leaf chewers (combinations) induced the JA-responsive gene *VSP2* with the only exception of plants attacked by *M. brassicae*, in which expression of *VSP2* was not different from control plants. The combination of *P. brassicae* – *P. cochleariae* caused the strongest induction of *VSP2* at 96h (Fig. S3b). The SA-responsive gene *PR1* was induced by attack of some (combination of) leaf chewers (Fig. S3d). Single attack by *P. cochleariae* or *A. rosae*, dual attack by *M. brassicae* – *A. rosae*, *M. brassicae* – *P. cochleariae*, or *A. rosae* – *P. cochleariae* and feeding by the four leaf-chewers induced expression of *PR1* at 96h (Fig. S4d). In contrast, feeding by phloem feeders and by each of the phloem-feeder species combinations did not induce any of the defence marker genes tested at 48 or 96h after attack (Fig. S4 & S5), except for *Myzus persicae* that induced *LOX2* expression 48h after feeding (Fig. S4).



In summary, feeding by the leaf chewers individually or in combination, induced the expression of the JA-biosynthesis and JA responsive genes *LOX2* and *VSP2*, respectively. Feeding by a mix of leaf-chewing and phloem-feeding insects, also induced *LOX2* and *VSP2*, but to a lesser extent. Feeding by the phloem feeders, or by combination of phloem feeders did not induce any of the marker genes tested but repressed the expression of the JA-responsive gene *VSP2* 48h after attack.

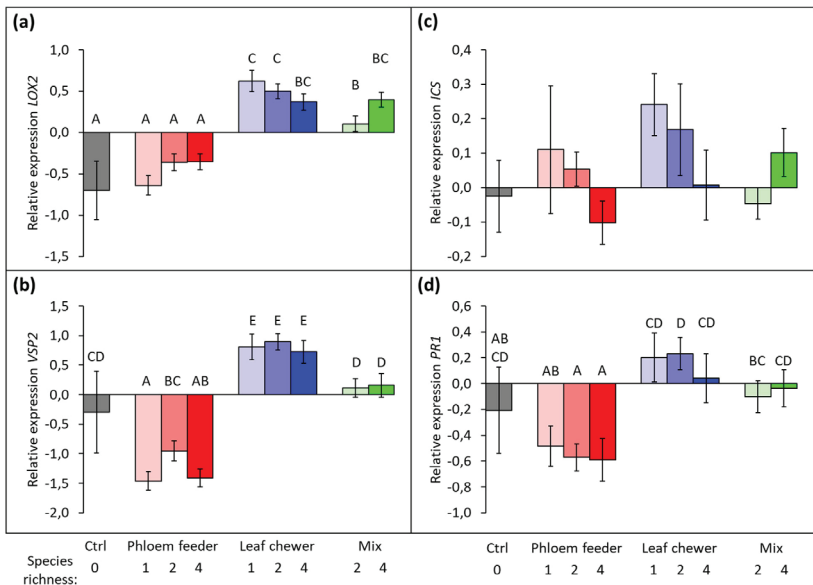


Figure 4. Relative gene expression of *Brassica nigra* leaves at 48h after infesting them with an increasing species richness (1, 2 or 4 species) of aphids, chewers, a mix of both or untreated plants (Ctrl). We measured the expression of JA-biosynthesis and JA-responsive genes *LOX2*, and *VSP2* (a) and (b), and the SA-biosynthesis and SA-responsive- genes *ICS* and *PR1* (c) and (d). Bars represent mean ± SE of log transformed data. Gene expression is relative to the expression level of two reference genes *GAPDH* and *SART1*. Bars not sharing letters are significantly different from each other (LM, post hoc LSD).



DISCUSSION

Multi-herbivore attack, species richness of herbivore attack as well as the composition of the species across feeding guilds influenced induced plant responses and resistance to subsequent herbivory. Plant responses to phloem-feeding insects promoted the growth of *P. xylostella*, whereas plant responses to leaf chewers reduced the performance of *P. xylostella* caterpillars. The higher the species richness of phloem feeders attacking the plant, the more vulnerable the plant became to larvae of the subsequent attacker *P. xylostella*. In contrast, induction by any combination of leaf-chewers regardless of species richness led to stronger resistance to *P. xylostella*. When the plant was attacked by a mix of phloem feeders and leaf chewers *P. xylostella* caterpillars performed equally well on plants induced with a mix of herbivore species from the two feeding guilds and on plants that had not received initial herbivore attack. Unexpectedly, in our study, the factor tray explained a substantial amount of variation. Plants sharing the same tray, were not only receiving the same herbivore, but could have affected each-other more strongly than plants in neighbouring trays through volatile or root communication via the shared pool of water. The compromise of plants in trays we had to make for feasibility of the experiment confounded biological effects associated with trays and those on our treatment level. Because trays were randomly distributed in the greenhouse having different neighbouring treatments, we deem it unlikely that volatile or root communication drives the patterns observed in our experiments. By using multiple species combinations per species richness level that were randomly placed in the greenhouse, independent blocks and experiments that all present similar findings, the most likely conclusion is the biological effect of species richness and feeding guild of the herbivores driving plant responses and resistance to *P. xylostella*.

The stronger susceptibility to larvae of *P. xylostella* after induction by a richer phloem feeder community suggests an additive effect of each phloem feeder species on suppressing plant responses to a leaf chewer. When feeding from the phloem sap, aphids inject effectors (i.e. aphid salivary proteins) that alter the plant primary and secondary metabolism (Mutti *et al.*, 2008; Giordanengo



et al., 2010; Elzinga & Jander, 2013; Züst & Agrawal, 2016; Ahman *et al.*, 2019; Jiang *et al.*, 2019). Excretion of salivary effectors by *M. persicae* has been found to reduce plant secondary metabolite production and callose deposition, benefitting aphid population growth (Elzinga *et al.*, 2014). The enhanced performance of *P. xylostella* on plants previously attacked by a higher species richness of phloem feeders, could potentially be attributed to a higher diversity of salivary effectors with more aphid species increasing the strength of the antagonistic effect of SA regulated resistance on JA responses. In our study, aphid feeding suppressed the expression of the JA-responsive gene *VSP2* 48h after attack. The JA pathway is a well-known defence pathway against insect herbivores and thus JA suppression is a potential explanation of the increased growth of *P. xylostella* on aphid-induced plants (Zarate *et al.*, 2007; Onkokesung *et al.*, 2010). The JA suppression, might be attributed to the aphids manipulating the host plant (Will *et al.*, 2007; Ahman *et al.*, 2019). However, we did not detect an aphid-induced expression of any of the two SA-marker genes studied nor the SA/JA crosstalk gene *WRKY70*. Thus, we do not have support to explain the aphid-induced changes based on the SA/JA negative crosstalk paradigm (Kroes *et al.*, 2015; Onkokesung *et al.*, 2016). Further research is needed to unravel the mechanisms behind the aphid-induced facilitation of the growth of *P. xylostella*.

In contrast with the phloem-feeder-induced vulnerability to *P. xylostella*, the growth of *P. xylostella* larvae was negatively affected by previous chewer attack with no role for species richness. Attack by a single leaf-chewer had a similar reduction on *P. xylostella* performance compared with treatments that were richer in leaf-chewer species. The absence of effect caused by species richness of chewers compared to the effect of richness caused by phloem feeders contrasts with the higher taxonomic richness used in the chewers than in the phloem feeders (all aphids). Thus, even with a wider phylogenetic richness of chewer species, combining these species did not strongly affect plant responses to chewing herbivores. Also in research on lima bean (*Phaseolus lunatus*), induced plant responses were not herbivore-specific and simultaneous attack by two chewers had a similar effect compared to single attack (Moreira *et al.*, 2015). On



wild radish plants (*Raphanus raphanistrum* and *R. sativus*) previously attacked by *P. xylostella* or *S. exigua* became more resistant to larvae of *S. exigua*, *Trichoplusia ni* (cabbage looper) and *Pieris rapae* (small cabbage butterfly) (Agrawal, 1999; Agrawal, 2000; Williams *et al.*, 2005; Rodriguez-Saona *et al.*, 2010). Interestingly in the study on wild radish, the strength and direction of the plant-mediated effect of chewer on chewer depended on the species involved, as previous infestation by *P. rapae* induced resistance only to *S. exigua* and *P. rapae*, and previous damage by *T. ni* (generalist) did not affect the performance of any of the herbivores (Agrawal, 2000). The fact that all leaf chewers and their combinations in our study equally affected plant resistance to a subsequent attacker only partly matches our molecular analysis, where we found that all chewers (and all chewer combinations) induce the JA-biosynthesis and JA-responsive genes *LOX2* and *VSP2* at the two time-points measured. However, single leaf chewer species induced these genes less strongly than a combination of two leaf chewer species. In *A. thaliana*, leaf chewers had a strong induction of the JA-responsive gene *VSP2*, and also became more resistant to subsequent chewers (Rasmann *et al.*, 2015). Additionally, we found that feeding by chewers induced expression of the SA-responsive gene *PR1*. Similarly, other studies reported induction of both SA and JA pathways upon leaf chewer feeding (Heidel & Baldwin, 2004; Li *et al.*, 2016a, b; Arena *et al.*, 2018; Kielkiewicz *et al.*, 2019). In *Brassica* interactions with chewing insects, specificity in response to leaf chewing herbivores may be low and suggests a less important role of Herbivore Associated Molecular Patterns (HAMP) and herbivore specific effectors than found for other plant-insect interactions (Erb & Reymond, 2019).

Plants simultaneously attacked by leaf chewers plus phloem feeders show similar resistance to larvae of *P. xylostella* compared to plants that did not receive herbivory. This suggests that the effect of aphid-induced vulnerability cancels out the effect of chewer-induced resistance to larvae of *P. xylostella*. In line with our findings, cotton plants simultaneously attacked by the chewer *S. exigua* and by the phloem feeder *Bemisia tabaci* emitted lower amounts of herbivore-induced plant volatiles than plants that were only attacked by *S. exigua* (Rodriguez-



Saona *et al.*, 2003). Likewise, *S. exigua* oviposition was deterred on tomato plants damaged by chewers, but the deterrence disappeared on plants that were simultaneously damaged by aphids and chewers (Rodríguez-Saona *et al.*, 2005). In our study, the expression of the two JA-related genes investigated was lower on plants simultaneously attacked by aphids and chewers compared to plants only under chewer attack. Similarly, caterpillar feeding on milkweed (*Asclepias syriaca*) induced JA, cardenolides and latex, and this induction was substantially attenuated in the presence of aphids (Ali & Agrawal, 2014). Likewise, in potato plants (*Solanum tuberosum*) feeding by *M. persicae* induced solanine levels (potato secondary metabolite) and the induction disappeared upon feeding by the chewer *Leptinotarsa decemlineata* (Davidson-Lowe *et al.*, 2019). The effect of mixed feeding guild attacks may be different across plant species. For example, tomato plants simultaneously attacked by aphids and chewers showed a similar defence response compared to plants only damaged by chewers (Rodríguez-Saona *et al.*, 2005).

Our study revealed that species richness and trait composition of the attackers as a component of multi-herbivore attack may affect plant induced responses and resistance to subsequent herbivores. We show canalization of plant defences in which plant responses to an increasing species richness in phloem feeders induced vulnerability to larvae of *P. xylostella*. The canalization response suggests that initial attack determines the plant phenotype and reduces potential to convert an induced phenotype in a direction that maximizes resistance to subsequent attack (Agrawal, 2001; Viswanathan *et al.*, 2007; Poelman *et al.*, 2008; Utsumi *et al.*, 2010; Stam *et al.*, 2014). However, in cases of facing simultaneous attack by herbivores from different feeding guilds, *B. nigra* plants integrate responses to both feeding guilds, showing an intermediate phenotype. In our study, integration of plant responses to phloem feeders and leaf chewers cancels out the effect of chewer-induced resistance to larvae of *P. xylostella* to levels of resistance of plants that were not previously attacked by herbivores. Although our current study only scratches the surface of plant resistance strategies to multi-herbivore attack by revealing a role for species richness, it should be a



starting point to further explore the importance of herbivore traits in multi-herbivore attack such as their level of food plant specialisation, order and timing of arrival. Disentangling the role of individual species in the context of natural communities from community processes driven by biodiversity components of multi-species interactions per se may provide deeper understanding of how plant defences evolve under community complexity. Critical steps that should be taken are to evaluate whether plant plasticity is adaptive to specific and perhaps predictable sequences of attack or whether plants can rely on less specified plastic responses to retain fitness. This not only requires to move to challenging plants with complex orders of attackers in controlled greenhouse studies, but to match these patterns with their natural context and assess fitness outcomes in these interactions. Plants may be selected for induced responses that match the patterns of multi-herbivore attack in which the response to one herbivore should not compromise resistance to likely future attack (Mertens *et al.*, 2021). We need to identify the repertoire of defence strategies that plants may use in different ecological settings to understand evolution of plastic defence strategies. Our study also identifies how challenging it is to compromise experimental design to unravel these plant strategies, which should nonetheless be taken to make it feasible to explore the scope of plant plasticity to multi-herbivore attack.

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AUTHOR CONTRIBUTIONS M.F.B., M.D. and E.H.P. planned and designed the study. M.F.B. collected most data. M.E.B. collected data on insect performance for approach 1 for the performance of *P. xylostella* on plants induced by leaf-chewer and mixed herbivores and together with M.F.B. collected data for the gene expression experiment. S.N.K. collected data on insect performance for approach 1 for the performance of *P. xylostella* on plants induced by phloem feeders. J.B. designed the primers and assisted during the gene expression experiment. G.G. carried out the statistical analyses. M.F.B., M.D. and E.H.P wrote the manuscript.



SUPPORTING INFORMATION

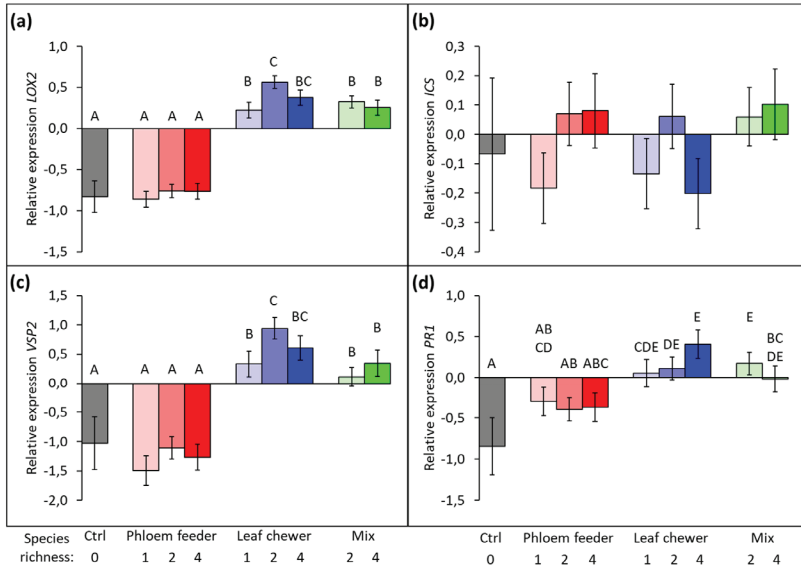


Fig. S1. Relative gene expression of *Brassica nigra* leaves at 96h after infesting them with an increasing species richness (1, 2 or 4 species) of aphids, chews, a mix of both or untreated plants (Ctrl). We measured the expression of JA-biosynthesis and JA-responsive genes *LOX2*, and *VSP2* (a) and (b), and the SA-biosynthesis and SA-responsive genes *ICS* and *PR1* (c) and (d). Bars represent mean \pm SE of log transformed data. Gene expression is relative to the expression level of two reference genes *GAPDH* and *SAR1A*. Bars not sharing letters are significantly different from each other (LM, post hoc LSD).



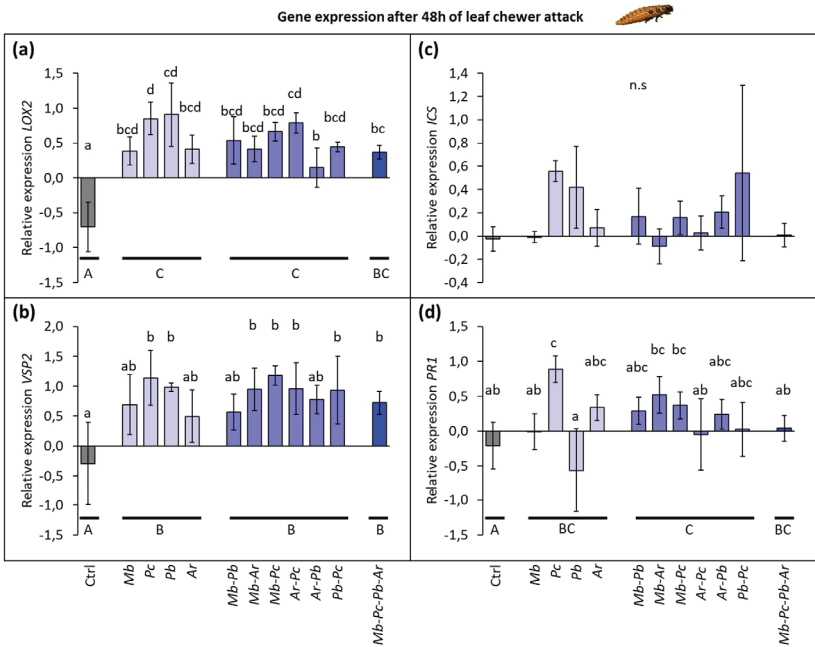


Fig. S2. Relative gene expression of *Brassica nigra* leaves at 48h after infesting them with an increasing species richness (1, 2 or 4 species) of the leaf chewers *Mamestra brassicae* (*Mb*), *Phaedon cochleariae* (*Pc*), *Pieris brassicae* (*Pb*), *Athalia rosae* (*Ar*) alone (Chew.1), in pairs of 2 species (Chew.2), the four species (Chew.4) or untreated plants (Ctrl). We measured the expression of JA biosynthesis and JA responsive genes *LOX2*, and *VSP2* (a) and (b), and the SA-biosynthesis and SA-responsive genes *ICS* and *PR1* (c) and (d). Bars represent mean \pm SE of log transformed data. Gene expression is relative to the expression level of two reference genes *GAPDH* and *SAR1A*. Bars not sharing letters are significantly different from each other (LM, post hoc LSD). Upper case letters above the bars represent differences between treatments. "n.s" not significant. Lower case letters below bar-groups represent differences between species richness levels.



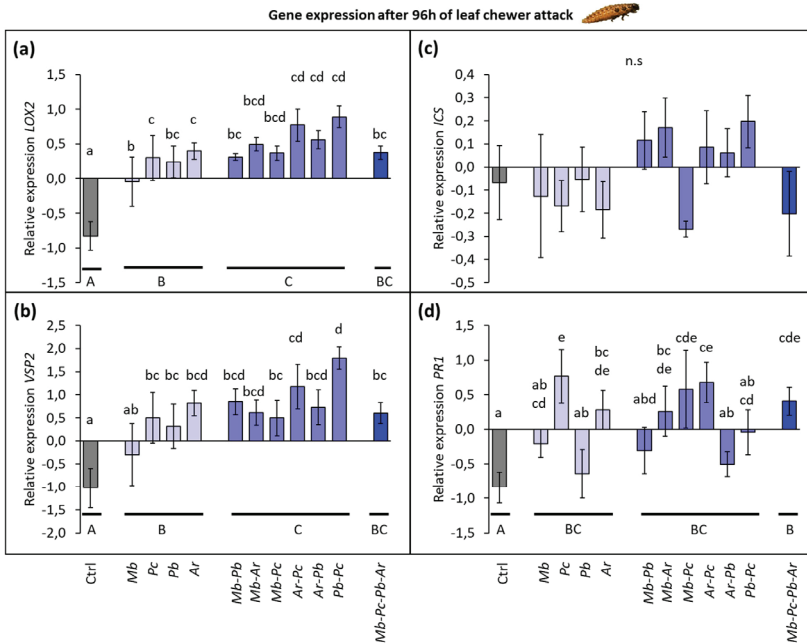


Fig. S3. Relative gene expression of *Brassica nigra* leaves at 96h after infesting them with an increasing species richness (1, 2 or 4 species) of the leaf chewers *Mamestra brassicae* (*Mb*), *Phaedon cochleariae* (*Pc*), *Pieris brassicae* (*Pb*), *Athalia rosae* (*Ar*) alone (Chew.1), in pairs of 2 species (Chew.2), the four species (Chew.4) or untreated plants (Ctrl). We measured the expression of JA-biosynthesis and JA-responsive genes *LOX2*, and *VSP2* (a) and (b), and the SA-biosynthesis and SA-responsive genes *ICS* and *PR1* (c) and (d). Bars represent mean \pm SE of log transformed data. Gene expression is relative to the expression level of two reference genes *GAPDH* and *SAR1A*. Bars not sharing letters are significantly different from each other (LM, post hoc LSD). "n.s." not significant. Upper case letters above the bars represent differences between treatments. Lower case letters below bar-groups represent differences between species richness levels.



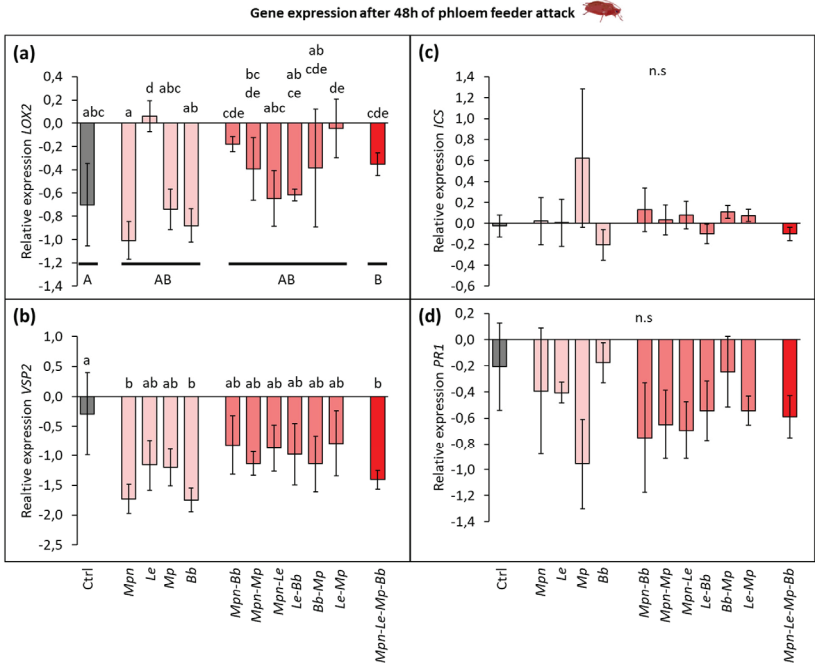


Fig. S4. Relative gene expression of *Brassica nigra* leaves at 48h after infesting them with an increasing species richness (1, 2 or 4 species) of the phloem-feeding aphids *Myzus persicae* sub. *nicotianae* (*Mpn*), *Lipaphis erysimi* (*Le*), *Myzus persicae* (*Mp*), *Brevicoryne brassicae* (*Bb*), alone (Phlo.1), in pairs of 2 species (Phlo.2), the four species (Phlo.4) or untreated plants (Ctrl). We measured the expression of JA-biosynthesis and JA-responsive genes *LOX2*, and *VSP2* (a) and (b), and the SA-biosynthesis and SA-responsive genes *ICS* and *PR1* (c) and (d). Bars represent mean \pm SE of log transformed data. Gene expression is relative to the expression level of two reference genes *GAPDH* and *SART1*. Bars not sharing letters are significantly different from each other (LM, post hoc LSD). "n.s" not significant. Upper case letters above the bars represent differences between treatments. Lower case letters below bar-groups represent differences between species richness levels.



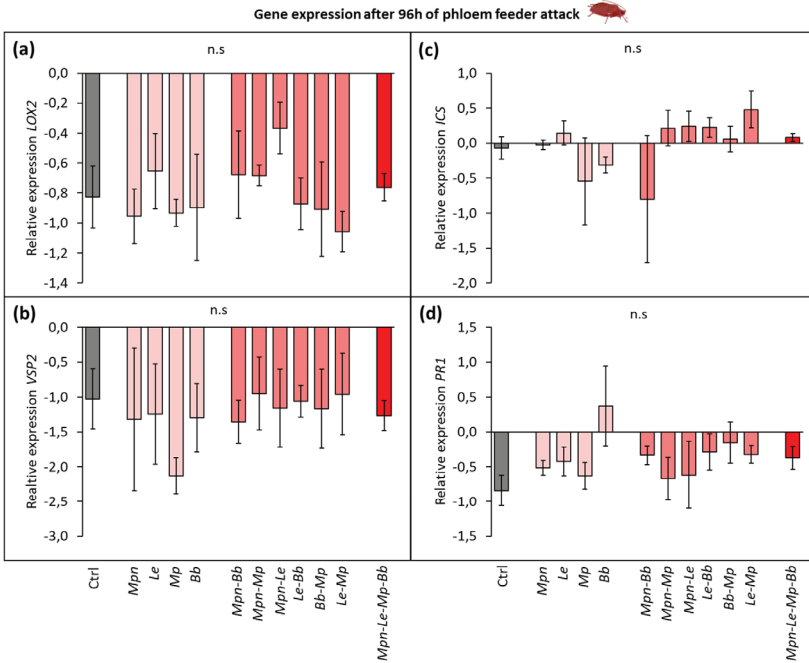


Fig. S5. Relative gene expression of *Brassica nigra* leaves at 96h after infesting them with an increasing species richness (1, 2 or 4 species) of the phloem feeding aphids *Myzus persicae* sub. *nicotianae* (Mpn), *Lipaphis erysimi* (Le), *Myzus persicae* (Mp), *Brevicoryne brassicae* (Bb), alone (Phlo.1), in pairs of 2 species (Phlo.2), the four species (Phlo.4) or untreated plants (Ctrl). We measured the expression of JA-biosynthesis and JA-responsive genes *LOX2*, and *VSP2* (a) and (b), and the SA-biosynthesis and SA-responsive genes *ICS* and *PR1* (c) and (d). Bars represent mean \pm SE of log transformed data. Gene expression is relative to the expression level of two reference genes *GAPDH* and *SAR1A*. Bars not sharing letters are significantly different from each other (LM, post hoc LSD). "n.s." not significant. Upper case letters above the bars represent differences between treatments. Lower case letters below bar-groups represent differences between species richness levels.



Table S1. Overview of insect combinations for the performance mix species richness experiment for approach 1. showing the number of plant replicates per species richness. We prepared a total of eight plants for each individual insect combination. For Mix.2 we did all the possible insect combinations. For Mix.4 we selected 12 out of the 36 possible combinations (underlined and bold). We did two consecutive sub experiments, preparing half of the replicates for each sub experiment. *Bb*= *Brevicoryne brassicae*, *Mp*= *Myzus persicae*, *Mpn*= *Myzus persicae* sub. *nicotianae*, *Le*= *Lipaphis erysimi*, *Ar*= *Athalia rosae*, *Pb*= *Pieris brassicae*, *Pc*= *Phaedon cochleariae*, *Mb*=*Mamestra brassicae*.

Con.0 1 treatment 10 plants	Mix.2 16 treatments 128 plants		Mix.4 12 treatments 96 plants	
Ctrl	<i>Bb-Mb</i>	<i>Mpn-Mb</i>	<u><i>Bb-Mp-Mb-Ar</i></u>	<i>Mp-Mpn-Mb-Ar</i>
	<i>Bb-Ar</i>	<i>Mpn-Ar</i>	<i>Bb-Mp-Mb-Pb</i>	<u><i>Mp-Mpn-Mb-Pb</i></u>
	<i>Bb-Pb</i>	<i>Mpn-Pb</i>	<i>Bb-Mp-Mb-Pc</i>	<i>Mp-Mpn-Mb-Pc</i>
	<i>Bb-Pc</i>	<i>Mpn-Pc</i>	<i>Bb-Mp-Ar-Pb</i>	<i>Mp-Mpn-Ar-Pb</i>
			<i>Bb-Mp-Ar-Pc</i>	<u><i>Mp-Mpn-Ar-Pc</i></u>
		<u><i>Bb-Mp-Pb-Pc</i></u>	<i>Mp-Mpn-Pb-Pc</i>	
	<i>Mp-Mb</i>	<i>Le-Mb</i>	<i>Bb-Mpn-Mb-Ar</i>	<i>Mp-Le-Mb-Ar</i>
	<i>Mp-Ar</i>	<i>Le-Ar</i>	<i>Bb-Mpn-Mb-Pb</i>	<i>Mp-Le-Mb-Pb</i>
	<i>Mp-Pb</i>	<i>Le-Pb</i>	<u><i>Bb-Mpn-Mb-Pc</i></u>	<u><i>Mp-Le-Mb-Pc</i></u>
	<i>Mp-Pc</i>	<i>Le-Pc</i>	<u><i>Bb-Mpn-Ar-Pb</i></u>	<u><i>Mp-Le-Ar-Pb</i></u>
			<i>Bb-Mpn-Ar-Pc</i>	<i>Mp-Le-Ar-Pc</i>
			<i>Bb-Mpn-Pb-Pc</i>	<i>Mp-Le-Pb-Pc</i>
			<i>Bb-Le-Mb-Ar</i>	<u><i>Mpn-Le-Mb-Ar</i></u>
			<u><i>Bb-Le-Mb-Pb</i></u>	<i>Mpn-Le-Mb-Pb</i>
			<i>Bb-Le-Mb-Pc</i>	<i>Mpn-Le-Mb-Pc</i>
			<i>Bb-Le-Ar-Pb</i>	<i>Mpn-Le-Ar-Pb</i>
			<u><i>Bb-Le-Ar-Pc</i></u>	<i>Mpn-Le-Ar-Pc</i>
			<i>Bb-Le-Pb-Pc</i>	<u><i>Mpn-Le-Pb-Pc</i></u>



Table S2. Overview of plant replicates per treatment and per species richness within each feeding guild for the approach 2: direct comparison of *Plutella xylostella* performance on phloem feeder, chewer and mixed herbivore induced *Brassica nigra* plants. Leaf chewers: Mb= *Mamestra brassicae*, Ar= *Athalia rosae*, Pb= *Pieris brassicae*, Pc= *Phaedon cochleariae*. Phloem feeders: Bb= *Brevicoryne brassicae*, Mp= *Myzus persicae*, Mpn= *Myzus persicae* sub. *nicotianae*, Le= *Lipaphis erysimi*.

Group	Species richness	Treatment	Rep.	Replicates group
Control	0	Con.0	<u>24</u>	24
Chewer	4	Chew. 4	24	24
Phloem feeder	4	Phlo. 4	24	<u>24</u>
		<i>Bb-Mp-Mb-Ar</i>	2	
		<i>Bb-Mp-Pb-Pc</i>	2	
		<i>Bb-Mpn-Mb-Pc</i>	2	
		<i>Bb-Mpn-Ar-Pb</i>	2	
		<i>Bb-Le-Mb-Pb</i>	2	
		<i>Bb-Le-Ar-Pc</i>	2	
Mix	4	<i>Mp-Mpn-Mb-Pb</i>	2	24
		<i>Mp-Mpn-Ar-Pc</i>	2	
		<i>Mp-Le-Mb-Pc</i>	2	
		<i>Mp-Le-Ar-Pb</i>	2	
		<i>Mpn-Le-Mb-Ar</i>	2	
		<i>Mpn-Le-Pb-Pc</i>	2	



Table S3. Primer sequences for the molecular analysis of *Brassica nigra* genes of interest and reference genes.

Gene	Pathway	AT	Sequence (Forward, reverse)
<i>LIPOXIGENASE 2 LOX2</i>	JA biosynthesis	62	TGCTCGTGCACGCCAGAGTC AGCCAGCCCCCTGCTGATGA
<i>VEGETATIVE STORAGE PROTEIN 2 VSP2</i>	JA responsive	58	TCTACGCCAAAGGACTTG CTCWGTCCCGTATCCATATTGAG
<i>ISOCHORISMATE SYNTHASE ICS</i>	SA biosynthesis	58	GCTTGACAGTTACAGAG CACGCTCTATCTCCATATCAC
<i>PATHOGENESIS-RELATED PROTEIN 1 PR-1</i>	SA responsive	60	CGCCGACGGACTAAGAGGCG ACACCTCGTTTGCCACATCCA
<i>WRKY70</i>	SA crosstalk	62	ATGCTTCHTGYGACAACGAC TTTGTTCCTTGACCCTTG
<i>SECRETION ASSOCIATED RAS RELATED GTPASE 1A SART1A</i>	Reference	60	ATCTCTAGCCACCGTTCCT TTCTGACGATGCTGCACAT
<i>GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE GAPDH</i>	Reference	62	GGAGCTGCCAAGGCTGTCCG CCTTCAGATTCTCTTGATAGCC
<i>ACTIN-2 ACT-2</i>	Reference	62	ACATTGTGCTCAGTGGTGGA TCTGCTGGAATGTGCTGAGG
<i>BETA-TUBULINE B-TUB</i>	Reference	62	GTCAAGTCCAGCGTCTGTGA TCACACGCCTGAACATCTCC
<i>ELONGATION FACTOR-1 EF-1</i>	Reference	60	CGTCCCATCTCTGGATTGCG ACAACCATACCGGGCTTGAG
<i>PEROXIDASE 4 PER4</i>	Reference	62	TATCTCTGCAGCCTCTCA ACACACAGACTGAAGCGTCC



Table S4. Variance components of the random factors of the statistical models used for testing for differences on performance of *P. xylostella*.

Variance components	With tray			Without tray		
	s ²	Wald Z	P	s ²	Wald Z	P
Block	0.00901	1.14	0.1269	0.00920	1.19	0.1167
Tray	0.00808	3.87	P<0.0001			
Plant	0.00223	2.22	0.0131	<u>0.00715</u>	5.45	P<0.0001
Error(aphids)	0.08366	20.3	P<0.0001	0.08194	20.10	P<0.0001
Error(chewers)	0.05074	22.74	P<0.0001	0.05096	22.51	P<0.0001
Error(mix)	0.09018	23.27	P<0.0001	0.09107	22.94	P<0.0001



Table S5. Significance differences of *Plutella xylostella* weight on plants previously attacked by a mix of leaf chewers and phloem feeders for approach 1. Insect combinations not sharing letters have a significant different effect on *Plutella xylostella* growth (MLM, Tukey post-hoc). Combination of inducers that affected *P. xylostella* growth (compared to control, untreated plants) are marked in bold and with an asterisk. Chewers: *Mamestra brassicae* (Mb), *Phaedon cochleariae* (Pc), *Pieris brassicae* (Pb), *Athalia rosae* (Ar). Phloem feeding aphids *Myzus persicae* sub. *nicotianae* (Mpn), *Lipaphis erysimi* (Le), *Myzus persicae* (Mp), *Brevicoryne brassicae* (Bb).

Treatment	Significance
Control	bcde
<i>Bb-Ar</i>	ab
<i>Bb-Mb</i>	abcde
<i>Bb-Pb</i>	cdef
<i>Bb-Pc</i>	abcd
<i>Le-Ar</i>	abc
<i>Le-Mb</i>	cdef
<i>Le-Pb</i>	abcd
<i>Le-Pc</i>	abc
<i>Mp-Ar</i>	bcde
<i>Mp-Mb</i>	bcdefg
<i>Mpn-Ar*</i>	fg
<i>Mpn-Mb</i>	bcde
<i>Mpn-Pb</i>	bcdefg
<i>Mpn-Pc</i>	cdef
<i>Mp-Pb</i>	cdef
<i>Mp-Pc</i>	bcdefg
<i>Bb-Le-Ar-Pc</i>	cdef
<i>Bb-Le-Pb-Mb</i>	ab
<i>Bb-Mp-Mb-Pc</i>	ef
<i>Bb-Mpn-Ar-Mb</i>	def
<i>Bb-Mpn-Pb-Pc</i>	bcdef
<i>Bb-Mp-Pb-Ar*</i>	a
<i>Mp-Le-Ar-Mb</i>	cdef
<i>Mp-Le-Pb-Pc</i>	abcde
<i>Mp-Mpn-Ar-Pc</i>	abc
<i>Mp-Mpn-Pb-Mb</i>	bcdef
<i>Mpn-Le-Mb-Pc*</i>	f
<i>Mpn-Le-Pb-Ar</i>	bcdef





CHAPTER 5
**Herbivore species richness influences
plant interactions with an herbivorous insect,
its natural enemies, and plant reproduction**

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Daan Mertens; Erik H. Poelman

* These authors contributed equally to this article

ABSTRACT

A main challenge in ecology is to assess how loss of species and consequently species interactions affect ecosystem stability. This knowledge is especially relevant within the context of insect decline that planet Earth faces. Here, we studied how insect herbivore species richness and trait composition of these species affect plant interactions with other insect community members (i.e. a herbivore, its parasitoids and pollinators) and whether these interactions affect plant fitness. We show that herbivore richness, its composition of functional traits and specific herbivore species affected the abundance of *Plutella xylostella*, the parasitism rate of *P. xylostella* and pollinator visitation to *Brassica nigra* plants. The composition of the introduced herbivore community affected plant fitness directly via reducing plant biomass that predicted resource investment into reproductive organs and these effects were stronger than fitness consequences of herbivore-induced plant-mediated interactions with other community members. Fitness effects of indirect interactions often included a network of multiple organisms and could be mediated by plant traits. All of these interactions involved effects of herbivores on pollinator visitation that closely correlated with the total number of seeds produced by a plant. For a species-rich community of phloem feeders as well as for the leaf chewer rich community seed set was reduced via a chain of interactions that reduced pollinator visitation. The effects of herbivore richness and functional traits in herbivore communities on plant interactions with subsequent herbivores, parasitoids and pollinators show how complex interaction networks on individual plants may determine plant fitness.



INTRODUCTION

Planet Earth is facing rapid changes in ecosystem dynamics and species interactions. Important drivers of these changes are human overpopulation, climate change, habitat loss and species extinction (Tylianakis *et al.*, 2006, 2008). The loss of integral parts of ecosystems, such as the worldwide insect decline, has caused rising concerns (Althaus *et al.*, 2021; Hallmann *et al.*, 2017; Halsch *et al.*, 2020; Harvey *et al.* 2020; Wagner *et al.*, 2021; Warren *et al.*, 2020). An inherent consequence of species loss is a reduction in the number of ecological interactions which may result in reduced ecosystem stability and secondary extinctions (Soliveres *et al.*, 2016; Tylianakis *et al.*, 2008). Experimental and modelling studies have identified that loss of individual species in insect communities not only has cascading effects on other community members (Sanders *et al.*, 2013, 2018), but also affects plant reproductive success (Lankau & Strauss, 2008; Soper Gorden & Adler, 2018). The cascading effects of interaction loss on plant fitness signals evolutionary consequences of species loss for plant trait selection (Zeng & Wiens, 2020). To understand and predict changes in selection regimes on plant traits by declines of organisms of higher trophic levels, a major challenge is identifying which insect groups and interactions more strongly affect plant fitness. This includes exploring direct and indirect interactions among antagonistic and mutualistic community members that may further modulate fitness outcomes of plant interactions with their insect community (Ando *et al.*, 2017; Poelman & Kessler, 2016; Rusman *et al.* 2019a).

In terrestrial ecosystems, plants are at the basis of many food chains being the food source for a species-rich community of insect herbivores (Schoonhoven, van Loon & Dicke, 2005) with 85% of plants also providing pollen and nectar to a diversity of flower-visiting insects (Ollerton *et al.*, 2011). Consequently, plants are central in a network of species that interact directly or indirectly via the plant (Ohgushi, 2005). The high diversity of organisms interacting with a plant creates a playground of interacting selective agents on the plant (e.g. herbivores, parasitoids or pollinators, abiotic stress and competition for resources with other plants). Due to the complexity of the plant-associated



network, prediction of selection on plant traits is not straightforward (Strauss, 2014). Indeed, diffuse evolution of plant traits is often observed, where selection on traits is not only explained by one-to-one species interactions, but rather is the effect of the network of interactions (Strauss, 2014; Poelman & Kessler 2016). Important selective agents on plant traits are herbivores, natural enemies of the herbivores and pollinators. Herbivores can directly affect plant fitness by consuming photosynthetic plant tissues, flowers and/or seeds and indirectly by altering the attraction of other herbivores, natural enemies of the herbivores and by altering pollinator visitation (Grass *et al.*, 2018; Rusman *et al.* 2020). Natural enemies of the herbivores, such as parasitoids and predators, can have positive direct effects on plant fitness by reducing herbivore pressure (Bustos-Segura *et al.*, 2020) and indirect effects by altering herbivore, omnivore and pollinator behaviour. Interactions with pollinators strongly affect plant fitness directly, because pollinators mediate plant reproduction. The increase in seed number may in turn attract seed-feeding herbivores (Pérez-Barrales *et al.*, 2013; Steenhuisen & Johnson, 2012). Thus, direct and indirect interactions between herbivores and other community members affect the insect community associated with a plant, including other herbivores (Poelman *et al.*, 2008), parasitoids (Blubaugh *et al.*, 2018; Bukovinszky *et al.*, 2012), pollinators (Chrétien *et al.*, 2021; Rusman *et al.*, 2018), and can affect plant fitness (Cozzolino *et al.*, 2015; Soper Gordon & Adler, 2018). However, it is poorly understood how plants maximise fitness when defending against attack by diverse assemblages of herbivores while maintaining the attraction of parasitoids/predators and pollinators.

Here, we study how herbivore species richness and its trait composition affect plant interactions with its insect community and whether these interactions affect plant fitness. We conducted a manipulative field study where we subjected *Brassica nigra* plants to attack by an insect herbivore community with increasing species richness from one and/or two feeding guilds (i.e. phloem feeders, leaf chewers or a mix of both). Recent studies identified that *B. nigra* plants respond differentially to attack by different herbivore feeding



guilds as well as the number of species attacking the plant (Fernández de Bobadilla *et al.*, 2021), and that indirect interactions due to herbivore-induced plant responses affect plant reproductive success (Pashalidou *et al.*, 2015; Rusman *et al.*, 2018, 2020). We specifically studied how the composition of the introduced herbivore community affected a) plant colonisation by the herbivore *Plutella xylostella*, one of the most prevalent herbivores attacking *B. nigra* plants (Mertens *et al.*, 2021); b) top-down control of this herbivore population by parasitoids; c) visitation of flowers by pollinators; and d) how these interactions collectively affect plant fitness in terms of seed production. We examined the relations between the different community members and effects of varying levels of herbivore richness and feeding guild by applying structural equation modelling (SEM). This allowed us to partition the direct and indirect effects of different community members on plant fitness, and how early-season herbivore attack alters these relationships. We consider as direct effects those where initial herbivory induces plant phenotypic changes that result in effects on plant fitness, whereas indirect effects are herbivore-induced effects on other recorded community members that have fitness consequences. By assessing the direct and indirect effects of herbivore community composition on important members of a plant-associated insect community this work sheds light on which interactions more strongly affect plant fitness, and potentially impose selection on plant traits.

MATERIALS AND METHODS









Plants and insects

Seeds of black mustard (*B. nigra*, Brassicales: Brassicaceae) were obtained from a natural population in the vicinity of Wageningen, The Netherlands (51° 57' 32" N, 5° 40' 23" E). Seeds were sown on peat soil (Lentse Potgrond) and germinated in a greenhouse at 22 ± 2 °C, 60-70 % RH and 16:8 h L:D. One week after germination, the plants were transplanted into peat soil cubes and planted in the field when three weeks old. One week before planting in the field, the plants were acclimatized to field conditions in an open greenhouse (i.e. without climate control).



Eight insect species that commonly attack *B. nigra* were used to manipulate the composition of the starting herbivore community on individual plants (Table 1). We used four chewing herbivore species: the turnip sawfly, *Athalia rosae* (*Ar*) (Hymenoptera: Tenthredinidae); the mustard leaf beetle, *Phaedon cochleariae* (*Pc*) (Coleoptera: Chrysomelidae); the cabbage moth, *Mamestra brassicae* (*Mb*) (Lepidoptera: Noctuidae); and the large cabbage white, *Pieris brassicae* (*Pb*) (Lepidoptera: Pieridae). We used four phloem-feeding herbivore species: the cabbage aphid, *Brevicoryne brassicae* (*Bb*); the mustard aphid, *Lipaphis erysimi* (*Le*); the green peach aphid, *Myzus persicae* (*Mp*); and the tobacco aphid, *Myzus persicae* sub. *nicotianae* (*Mpn*) (all Hemiptera: Aphididae) (Table 1). *Mamestra brassicae*, *P. brassicae*, *B. brassicae* and *P. xylostella* were reared on Brussels sprouts plants (*Brassica oleracea* L. var. *gemmifera* cv. Cyrus). *Myzus persicae*, *M. persicae* sub. *nicotianae*, *L. erysimi*, *A. rosae* and *P. cochleariae* were reared on radish (*Raphanus sativus*). All insects were obtained from the stock rearing of the Laboratory of Entomology, Wageningen University kept under greenhouse conditions (22 ± 2 °C, 60–70 % RH and 16:8 h L:D).

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

Species (Abbreviation)	Picture	Order	Feeding guild
<i>Athalia rosae</i> (<i>Ar</i>)		Hymenoptera	Leaf chewer
<i>Mamestra brassicae</i> (<i>Mb</i>)		Lepidoptera	Leaf chewer
<i>Phaedon cochleariae</i> (<i>Pc</i>)		Coleoptera	Leaf chewer
<i>Pieris brassicae</i> (<i>Pb</i>)		Lepidoptera	Leaf chewer
<i>Brevicoryne brassicae</i> (<i>Bb</i>)		Hemiptera	Phloem feeder
<i>Lipaphis erysimi</i> (<i>Le</i>)		Hemiptera	Phloem feeder
<i>Myzus persicae</i> (<i>Mp</i>)		Hemiptera	Phloem feeder
<i>Myzus persicae</i> sub. <i>nicotianae</i> (<i>Mpn</i>)		Hemiptera	Phloem feeder



Plant treatments

The effect of species richness and trait composition and trait richness of the herbivore community on plant interactions with herbivores, parasitoids and pollinators and on plant seed production were studied in the Droevendaal experimental fields of Wageningen University (51° 59' 26.5" N, 5° 39' 50.5" E) during the summer of 2019. We planted 800 plants divided over 5 blocks: each block was a field of 21x17m with 160 plants planted on the same day at 1.5m distance from each other. Blocks were planted separately every two weeks between the 6th of May and 1st of July 2019 (Fig. 1).

Two days after planting in the field, we infested plants with different herbivore communities by varying species richness of herbivores (one, two or four herbivore species) and their trait composition in terms of the feeding guilds they belong to (leaf-chewing, phloem-feeding, or a mix of both). This resulted in 13 treatments: control plants without herbivore treatment (Con-0), four different treatments with one chewing herbivore species (Chew-1), one treatment with the four chewing herbivore species combined (Chew-4), four different treatments with one phloem-feeding herbivore species (Phlo-1), one treatment with the four phloem-feeding herbivore species combined (Phlo-4), mixture of one chewing and one phloem-feeding herbivore species (Mix-2), mixture of two chewing and two phloem-feeding herbivore species (Mix-4). We excluded species richness of three species, because of the imbalance that these treatments would have in numbers of leaf-chewer and phloem-feeder species. In each block, we included 12 replicates per herbivore combination for the chewer and phloem feeder treatments (Chew-1, Chew-4, Phlo-1 Phlo-4). For the Mix-2 and Mix-4 treatments, we included one replicate for each herbivore combination: Mix-2 resulted in 16 insect combinations, while for Mix-4 a selection of 12 of the 36 species combinations were included. Combinations were selected to have the largest differences between species combinations, while each herbivore is present the same number of times (6 times) (Fernández de Bobadilla *et al.*, 2021). For a complete list of all species combinations see Table 2. All treatments were randomised per block.



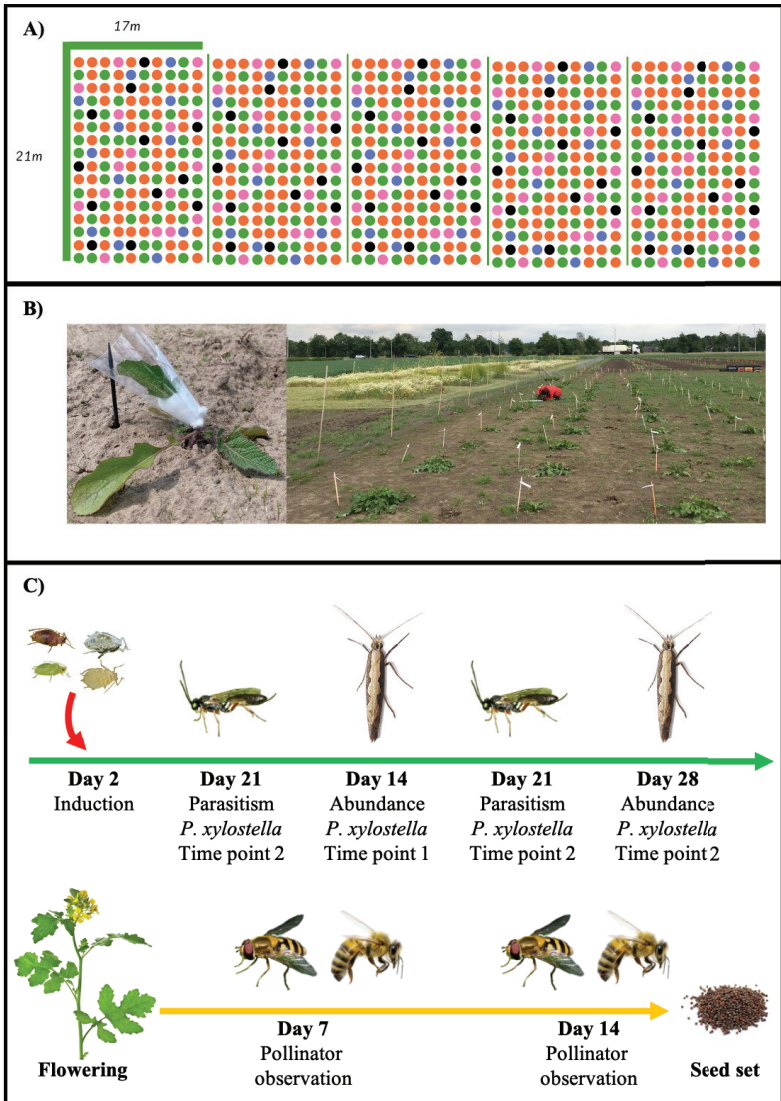


Figure 1. Experimental setup. A) Field setup: 5 blocks, each colour represents one herbivore treatment. B) Experiment time line of the measurements



Table 2. Overview of insect combinations for the mixed species richness treatments. For Mix-2 we did all the possible insect combinations. For Mix-4 we selected 12 out of the 36 possible combinations (underlined and bold). *Bb*= *Brevicoryne brassicae*, *Mp*= *Myzus persicae*, *Mpn*= *Myzus persicae* sub. *nicotianae*, *Le*= *Lipaphis erysimi*, *Ar*= *Athalia rosae*, *Pb*= *Pieris brassicae*, *Pc*= *Phaedon cochleariae*, *Mb*=*Mamestra brassicae*.

Mix-2 16 treatments		Mix-4 12 treatments	
<i>Bb-Mb</i>	<i>Mpn-Mb</i>	<u><i>Bb-Mp-Mb-Ar</i></u>	<i>Mp-Mpn-Mb-Ar</i>
<i>Bb-Ar</i>	<i>Mpn-Ar</i>	<i>Bb-Mp-Mb-Pb</i>	<u><i>Mp-Mpn-Mb-Pb</i></u>
<i>Bb-Pb</i>	<i>Mpn-Pb</i>	<i>Bb-Mp-Mb-Pc</i>	<i>Mp-Mpn-Mb-Pc</i>
<i>Bb-Pc</i>	<i>Mpn-Pc</i>	<i>Bb-Mp-Ar-Pb</i>	<i>Mp-Mpn-Ar-Pb</i>
		<i>Bb-Mp-Ar-Pc</i>	<u><i>Mp-Mpn-Ar-Pc</i></u>
		<u><i>Bb-Mp-Pb-Pc</i></u>	<i>Mp-Mpn-Pb-Pc</i>
<i>Mp-Mb</i>	<i>Le-Mb</i>	<i>Bb-Mpn-Mb-Ar</i>	<i>Mp-Le-Mb-Ar</i>
<i>Mp-Ar</i>	<i>Le-Ar</i>	<i>Bb-Mpn-Mb-Pb</i>	<i>Mp-Le-Mb-Pb</i>
<i>Mp-Pb</i>	<i>Le-Pb</i>	<u><i>Bb-Mpn-Mb-Pc</i></u>	<u><i>Mp-Le-Mb-Pc</i></u>
<i>Mp-Pc</i>	<i>Le-Pc</i>	<u><i>Bb-Mpn-Ar-Pb</i></u>	<u><i>Mp-Le-Ar-Pb</i></u>
		<i>Bb-Mpn-Ar-Pc</i>	<i>Mp-Le-Ar-Pc</i>
		<i>Bb-Mpn-Pb-Pc</i>	<i>Mp-Le-Pb-Pc</i>
		<i>Bb-Le-Mb-Ar</i>	<u><i>Mpn-Le-Mb-Ar</i></u>
		<u><i>Bb-Le-Mb-Pb</i></u>	<i>Mpn-Le-Mb-Pb</i>
		<i>Bb-Le-Mb-Pc</i>	<i>Mpn-Le-Mb-Pc</i>
		<i>Bb-Le-Ar-Pb</i>	<i>Mpn-Le-Ar-Pb</i>
		<u><i>Bb-Le-Ar-Pc</i></u>	<i>Mpn-Le-Ar-Pc</i>
		<i>Bb-Le-Pb-Pc</i>	<u><i>Mpn-Le-Pb-Pc</i></u>

In all herbivore communities the starting density consisted of four individuals for leaf chewer treatments and eight individuals for phloem feeder treatments, divided over one or four species. For example, Chew-1 included four individuals of the same leaf chewer species, while Chew-4 included one individual of each leaf chewer species. For the mixed treatments, we used two leaf-chewer individuals plus four phloem-feeder individuals, divided over two or four species. For chewing herbivores, first-instar larvae were placed on the youngest fully expanded leaf of the plant, while for phloem-feeding herbivores we used wingless adults. Insects could freely move and feed from their preferred feeding sites on the plant. The number of species and densities of inducers were based on field observations of insect communities of *B. nigra* plants in the same area (Mertens *et al.*, 2021).



Natural colonisation of *P. xylostella*

To investigate the effects of trait composition and species richness of the herbivore community on subsequent interactions with the herbivore community, we monitored natural colonisation of the herbivore *P. xylostella*. *Plutella xylostella* is one of the dominant herbivores colonising brassicaceous plants in the vegetative state. We recorded the number of larvae of *P. xylostella* that naturally colonised individual plants of each of the 13 herbivore community treatments including plants that were not experimentally infested with herbivores. Plants were checked for caterpillar presence at two time points, two and four weeks after planting them in the field. We did so by carefully checking each leaf for the presence of caterpillars of *P. xylostella* (L2-L4). To further support the field observations on caterpillar presence, we tested moth oviposition preference for plants infested with the different herbivore communities in a greenhouse setup (Fig. S1, Tables S1 and S3).

Parasitism of *P. xylostella*

To investigate the effects of trait composition and species richness of the herbivore community on the top-down control of *P. xylostella* by parasitoids, we monitored natural parasitism of caterpillars of *P. xylostella* on plants infested with each of the 13 herbivore community treatments including plants that were not infested with herbivores. We placed 15 L2-L3 *P. xylostella* larvae on a young leaf of each plant, nine and eighteen days after the initial induction. The leaf petiole was covered with some cotton and surrounded with a transparent plastic sheet shaped as a funnel to prevent the caterpillars from escaping (Fig. 1). The caterpillars were placed five days before assessing the abundance of *P. xylostella* (see previous section) to ensure that, in the rare case of a caterpillar escaping, these would have pupated and would not be counted as natural colonisation of *P. xylostella*. The caterpillars were placed at 10 a.m. and, two days later, at 5 p.m., caterpillars were recollected, transferred to Petri dishes and stored in a refrigerator (5 °C) or in a freezer (-20 °C) for longer storage until dissection. The caterpillars were dissected and checked for the presence of parasitoid eggs using a stereomicroscope (Olympus SZ51 KL-300 LED). The parasitoid eggs



or larvae were not identified to the species level. However, field studies show that the most common parasitoids of *P. xylostella* caterpillars in The Netherlands are *Diadegma* spp. (Bukovinszky *et al.*, 2004).

Pollinator visitation

To investigate effects of trait composition and species richness of the herbivore community on pollinator visitation, we recorded pollinator visitation on plants infested with each of the 13 herbivore community treatments including plants that were not infested with herbivores. Recordings were done at two time points: a) between 7 and 9 days and b) between 14 and 16 days after plants had started flowering. Pollinator visitation to a plant was monitored for 10 min. When a pollinator arrived to a plant and had contact with a flower, the identity of the pollinator and the number of flowers visited were recorded (Rusman *et al.*, 2018, 2020). The identity of other pollinators that visited the plant during this observation was recorded as well. If the same pollinator individual returned to the plant under observation after having visited a different plant, we scored that visit as a new visit (Rusman *et al.*, 2018, 2020). Recordings were done during the day (between 9 a.m. and 1 p.m., or 2 p.m. and 5 p.m.) only when weather conditions were favourable for pollinator activity (15–30 °C and wind speed ≤ 6 m/s (Rusman *et al.*, 2018, 2020), using a handheld computer (Psion Workabout Protm 3) programmed with The Observer XT software (version 10, Noldus Information Technology, Wageningen, The Netherlands).

Plant phenotype and seed production

To investigate the effects of herbivore-induced changes on plant phenotype and assess whether these changes affect plant interactions with the insect community, we measured multiple plant parameters: the number of leaves, plant height, plant diameter and percentage of leaf damage. Measurements were taken after assessing the abundance of *P. xylostella* (see previous section), using a tape measure. The percentage of leaf damage on a full plant scale was visually estimated on a percentage scale (0–100%).



Plants were monitored every 2-3 days for the start and end of the flowering period. Plants were considered flowering when the first flower opened and considered finished with flowering when all buds and flowers were gone, with only developing and ripe siliques remaining on flower stalks (Rusman *et al.*, 2020). We calculated the duration of flowering by subtracting the number of days needed to start flowering from the number of days to the termination of flowering (Rusman *et al.*, 2020).

Life-time seed production was assessed by seed number and weight. First harvesting date for each plant was selected before the first siliques would lose their seeds (Rusman *et al.*, 2018; 2020). At first harvest, we collected all ripe siliques and left immature siliques and flowers on the plant. Then, plants were checked weekly and all siliques were harvested when ripe. Siliques were stored in paper bags in a dry storage room until seeds were manually extracted from the siliques. We calculated the total number of seeds per plant by weighing 100 seeds, and the total weight of seeds harvested per plant (Rusman *et al.*, 2018, 2020). We estimated the total number of seeds by dividing total seed weight by the weight of 100 seeds and multiplied the result by 100. The weight of one seed was estimated by dividing the weight of 100 seeds by 100.

Statistical analysis

The effects of species richness and trait composition of the herbivore community were first analysed separately for plant interactions with *P. xylostella*, parasitoids, pollinators, and plant traits (flowering traits, seed production) using generalized linear mixed modelling, GL(M)M. For count data such as number of insects (e.g. number of herbivores, parasitoid eggs, or pollinators), flowers visited, days (from infestation, since flowering), and seed numbers, we used GL(M)M with a Poisson distribution and a log link function, or negative binomial distribution with a log link function to correct for overdispersion when necessary. For continuous data such as seed weight, we used linear (mixed) models with a Gaussian distribution and



identity link function or a Gamma distribution with a log link function if the data did not follow a normal distribution. Two models were fitted to the data: the first model included herbivore treatment and time point as fixed factors. The second model included herbivore species richness (treatments assigned to 0, 1, 2 or 4 species), herbivore feeding guild (treatments assigned to control, chewing, phloem-feeding, or combination), and time point as fixed factors. Non-significant interactions ($P > 0.05$) were excluded from the model. Random factors were selected using a backward approach: all random factors such as block, day (only for pollinators), time (morning or afternoon; only for pollinators) were added to the models and removed if they explained $< 3\%$ of the variation or were statistically non-significant ($P > 0.05$). Model assumptions were assessed graphically using the Pearson residuals against the fitted value by the models as well as against all the included covariates (i.e. fixed factors). Pairwise comparisons were evaluated using Tukey's *post hoc* test for significant variables ($P < 0.05$). For comparisons within treatment and combined herbivore species richness * feeding guild we used non-adjusted p values to cope with the high number of treatments. For comparisons within herbivore species richness and feeding guild we used adjusted p values. For number of parasitized *P. xylostella* recaptured, an offset with the natural logarithm of the total number of recaptured *P. xylostella* larvae was added to the model to account for differences in the total number of larvae recaptured per plant. Plants for which no *P. xylostella* larvae were recovered were not included in the analysis as they do not provide information on the prevalence of parasitism of *P. xylostella* larvae. Unfortunately, larvae from the fifth block were lost due to unexpected storage failure by malfunction of the freezer and could not be included in the analysis. For plant seed production, plants that produced $\leq 2,500$ or $> 40,000$ seeds were considered artifacts. Removing these outliers affected all treatments equally (no overall effect of herbivory; $p = 0.431$ for plants with 0 to 2,500 seeds). In addition, plants from block 5 were excluded due to extremely high mortality (almost 80%). We used the lme4 (Bates *et al.*, 2015),



multcomp (Hothorn *et al.*, 2008), lsmeans (Lenth, 2016), lmtest (Zeileis and Hothorn, 2002), emmeans (Russell, 2018), ggplot2 (Wickham, 2016) packages for these analyses.

We performed structural equation modelling (SEM) to examine the effects of the herbivore treatment on relations between plant traits and plant interactions with *P. xylostella* and their natural enemies, pollinators, and plant fitness. We built three models: one including each herbivore species treatment, one including herbivore species richness, and one including herbivore feeding guild. We did this to identify effects of individual herbivore species, as well as broader patterns based on herbivore richness or herbivore feeding guild. All included variables are displayed in figure 14. The variables height and radius were used as independent variables instead of dependent variables because of model identification problems. For parasitism rate and number of pollinators and flowers visited we only used data from time point 1, because time point 2 contained too few observations. We only used total pollinator visits and total flowers visited because including individual pollinator species/groups reduced the number of observations. All variables were checked for normality and homogeneity of variance, and if violated, variables were log-transformed. Since transformation did not solve assumption violations in all cases, we used the “Yuan-Bentler” test statistic. Good model fit was verified by inspecting the robust Test statistic ($P > 0.05$), CFI (> 0.9), RMSEA (< 0.08), RMSEA Low (< 0.01), RMSEA High (< 0.1), SRMR (< 0.08). We used the lavaan package for these analyses (Rossee 2012). All analyses were performed in R version 4.0.2 (R Core Team, 2020).

RESULTS

Natural colonisation of *Plutella xylostella* is affected by herbivore richness

The abundance of *P. xylostella* differed among plants induced by herbivore communities that differed in richness and species composition ($df = 1$, $\chi^2 = 4.67$, P



Table 3. Output of generalized linear (mixed) models showing the effects of different fixed factors (species richness, feeding guild, time point) on interactions of *Brassica nigra* plants with *Plutella xylostella* and its natural enemies, attractions and visitations by pollinators, flowering traits, and plant seed production. All random factors were initially included in the model, and factors which explained less than 3 percent variation or with a *P* - value above 0.05 were excluded from the model. Bold values indicate results where *P* ≤ 0.05. Italic values indicate results where *P* ≤ 0.1.

	Species richness (SR)			Feeding guild (FG)			Time point (TP)			SR*FG			SR*TP			FG*TP			SR*FG*TP			Block/Day (pollinators)		
	df	χ^2	<i>P</i>	df	χ^2	<i>P</i>	df	χ^2	<i>P</i>	df	χ^2	<i>P</i>	df	χ^2	<i>P</i>	df	χ^2	<i>P</i>	df	χ^2	<i>P</i>	df	χ^2	<i>P</i>
<i>Plutella xylostella</i> abundance	2	2.90	0.235	3	2.27	0.518	1	1.39	0.239	1	0.11	0.744	2	1.93	0.381	3	2.41	0.491	1	4.67	0.031	1	31.47	<0.001
<i>Plutella xylostella</i> parasitism	2	5.47	0.065	3	16.18	0.001	1	68.00	<0.001	-	-	-	-	-	-	-	-	-	-	-	-	-	28.57	<0.001
Number of pollinators	3	3.31	0.346	2	8.35	0.015	1	0.08	0.774	1	0.10	0.750	3	4.75	0.191	2	5.15	0.076	1	0.98	0.323	1	51.32	<0.001
Flowers visited by pollinators	3	1.83	0.608	2	4.79	0.091	1	1.02	0.313	1	3.74	0.053	3	3.90	0.272	2	0.76	0.684	1	0.01	0.937	1	24.25	<0.001
Time to flower	3	1.86	0.601	2	3.93	0.140	-	-	-	1	0.13	0.714	-	-	-	-	-	-	-	-	-	-	610.60	<0.001
Flowering time	3	2.00	0.573	2	1.85	0.397	-	-	-	1	0.00	0.984	-	-	-	-	-	-	-	-	-	-	-	-
Plant seed production	3	6.34	0.096	2	4.04	0.133	-	-	-	1	0.00	0.967	-	-	-	-	-	-	-	-	-	-	17.19	<0.001
Seed weight	3	11.40	0.010	2	6.89	0.032	-	-	-	1	4.18	0.041	-	-	-	-	-	-	-	-	-	-	154.70	<0.001



= 0.031, Table 3). These effects were restricted to *P. xylostella* abundance two weeks after induction of *B. nigra* plants and not observed within one week after induction (Fig. 2). We found fewer *P. xylostella* caterpillars on plants that did not receive initial herbivory and on plants attacked by four leaf chewers compared with the other induction treatments. Irrespective of species richness, phloem-feeder induction made the plants more attractive to *P. xylostella* compared to untreated plants. In contrast, in the case of leaf chewers the richness of attackers was important, as plants attacked by one chews species had a larger number of *P. xylostella* than plants attacked by four species of leaf chewers (Fig. 2). When plants were attacked by the highest species richness of herbivores (i.e. four species) the abundance of *P. xylostella* differed depending on the guild of the attacker. Plants attacked by four species of phloem feeders had the largest number of *P. xylostella*, followed by plants attacked by chewers and phloem feeders at the same time (i.e. Mix-4) and plants attacked by four species of chewers had the smallest number of *P. xylostella*. Likewise, in a greenhouse study, the oviposition preference of *P. xylostella* changed depending on the guild of the herbivores that had previously attacked the plant. *Plutella xylostella* laid more eggs on plants attacked by four species of phloem feeders compared with plants attacked by a mix of two phloem feeders and two chewers (i.e. Mix-4) ($P < 0.001$; Wilcoxon signed rank test, Fig. S2). In contrast to the overall influence of trait composition and species richness of the attacker community, the abundance of *P. xylostella* in the field was not affected by specific herbivores (Table 4). However, in a greenhouse study, chews identity affected the oviposition preference of *P. xylostella*. The moths laid fewer eggs on plants attacked by *M. brassicae* and more eggs on plants attacked by *P. cochleariae* compared to plants attacked by the four chews species ($P < 0.05$, Wilcoxon signed rank test, Fig. S3). The oviposition preference of *P. xylostella* was not affected by the identity of phloem feeders nor by the richness of species in a mix with chewers and phloem feeders (Fig S4, S5).



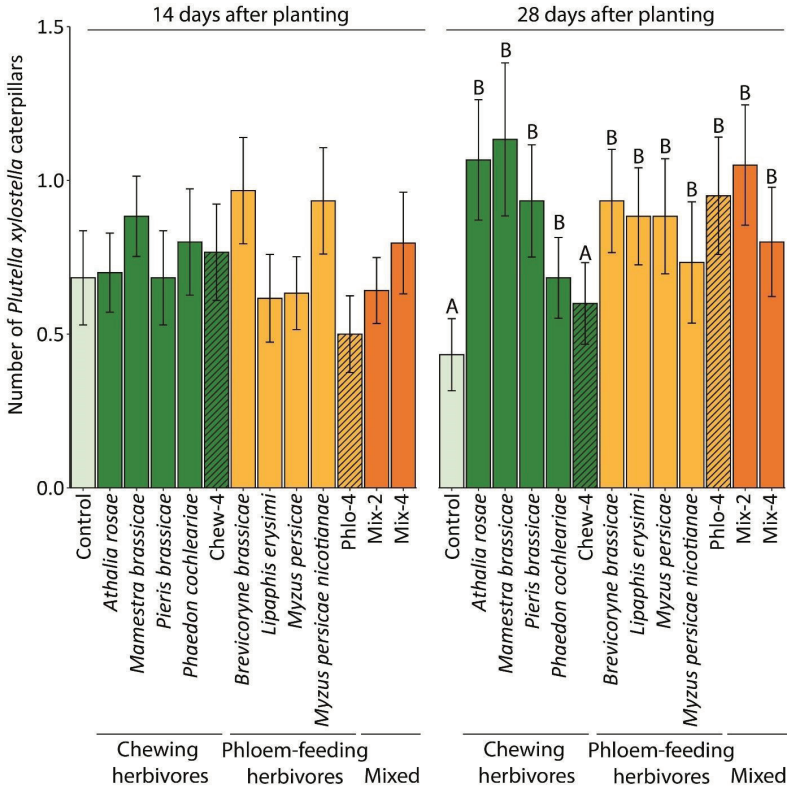


Figure 2. Abundance of *Plutella xylostella* larvae 14 and 28 days after planting on herbivore-treated- or on untreated plants. Bars represent the mean \pm the standard error of the mean. Capital letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments within a time point based on non-adjusted Tukey's post hoc tests.



Table 4. Output of generalized linear (mixed) models showing the effects of different fixed (herbivore species, time-point) factors on interactions of *Brassica nigra* plants with *Plutella xylostella* and its natural enemies, attraction and visitation by pollinators, flowering traits, and plant seed production. All random factors were initially included in the model, and factors which explained less than 3 percent variation or with a P -value above 0.05 were excluded from the model. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

	Herbivore treatment (Γ)			Time point (TP)			T*TP			Block			Day		
	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P	df	χ^2	P
<i>Plutella xylostella</i> abundance	12	11.99	0.446	1	1.44	0.231	12	14.99	0.242	1	32.25	<0.001	-	-	-
<i>Plutella xylostella</i> parasitism	12	23.26	0.026	1	67.55	<0.001	-	-	-	1	28.25	<0.001	-	-	-
Number of pollinators	12	23.58	0.023	1	0.11	0.744	12	19.31	0.087	-	-	-	1	42.72	<0.001
Flowers visited by pollinators	12	17.22	0.141	1	1.49	0.222	12	14.71	0.258	1	25.03	<0.001	-	-	-
Time to flower	12	11.26	0.507	-	-	-	-	-	-	1	610.24	<0.001	-	-	-
Flowering time	12	28.40	0.005	-	-	-	-	-	-	-	-	-	-	-	-
Number of seeds	12	21.48	0.044	-	-	-	-	-	-	1	14.31	<0.001	-	-	-
Seed weight	12	44.09	<0.001	-	-	-	-	-	-	1	158.06	<0.001	-	-	-



Parasitism of *Plutella xylostella* in the field is affected by the guild of initial attackers

The parasitism of *P. xylostella* was significantly different across feeding guild and time point (Feeding guild: $df = 3$, $\chi^2 = 16.18$, $P < 0.001$; time point: $df=1$ $\chi^2 = 67.99$, $P < 0.001$; Fig. 3, Table 3). The number of parasitised larvae was significantly higher on plants induced by a mixture of chewers and phloem feeders (i.e. Mix-4) than on plants attacked by one feeding guild alone (Fig. 3). Between leaf chewer and phloem feeder communities there was no difference in the number of parasitised *P. xylostella* larvae (Fig. 3). The species richness of herbivores attacking the plant marginally affected the parasitism of *P. xylostella* ($df = 2$, $\chi^2 = 5.47$, $P = 0.065$; Fig. 3 and Table 3). In addition to feeding guild and richness of the attacker community, specific herbivores affected the parasitism rate of *P. xylostella* ($df = 12$, $\chi^2 = 23.26$, $P = 0.026$; Fig. 4 and Table 3). The degree of *P. xylostella* parasitism was higher on plants attacked by *P. brassicae*, *M. persicae*, *M. persicae* sub. *nicotianae* and by a mix of two phloem feeders and two chewers (i.e. Mix-4) compared with untreated plants (Fig. 4). The parasitism on Mix-4 plants was higher than on plants induced by the four species of phloem feeders, and plants induced by the mix of the four species of leaf chewers. In addition to the herbivore treatments, the time of measurement also affected the parasitism rate of *P. xylostella* larvae which was higher nine days after initial induction, compared to 18 days after initial induction ($df = 1$, $\chi^2 = 67.55$, $P = 0.001$; Figs. 3 and 4, Tables 3 and 4).

Pollinator visitation in the field is affected by richness of initial attackers

We observed 4077 flowering plant visits by at least 21 insect groups/species (Table 5). Almost 80 percent of the visits were by two dominant species: *Eristalis tenax* and *Apis mellifera*. For the remaining visits we observed more than seven syrphid fly species, more than four bumblebee species, and several solitary bee, fly, butterfly and wasp species.



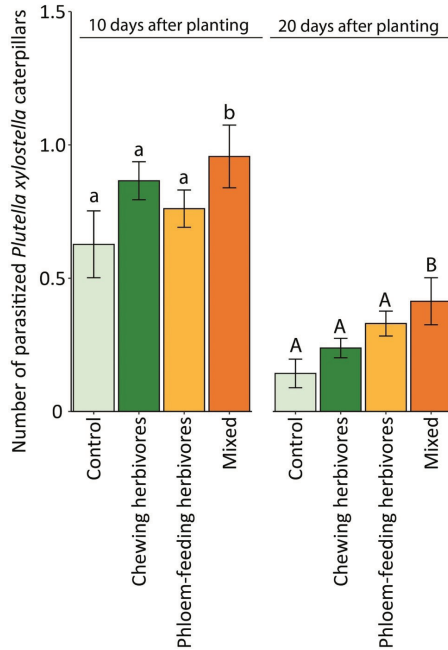


Figure 3. Number of parasitized *Plutella xylostella* larvae on *Brassica nigra* plants treated with different herbivores grouped by feeding guild and species richness. Plants were treated 2 days after planting or were left untreated at the two time points measured. Bars represent the mean \pm the standard error around the mean. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments within a time point based on adjusted Tukey's post hoc tests, small and capital letters were used for different time points.



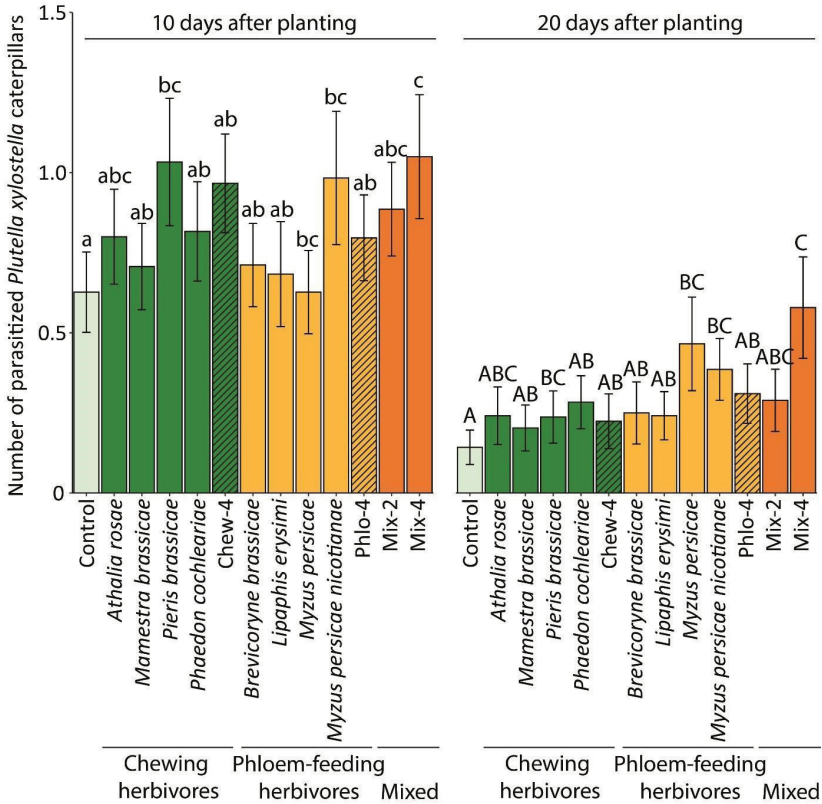


Figure 4. Number of parasitized *Plutella xylostella* larvae 10 or 20 days after planting on plants that received herbivore treatments or were left untreated. Bars represent the mean \pm the standard error around the mean. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments within a time point based on non-adjusted Tukey's post hoc tests, small and capital letters were used for different time points.



Table 5. Pollinator groups/species observed visiting flowers of *Brassica nigra*.

Pollinator groups	Species	Total number observed	Percentage of total visitors
Honeybees	<i>Apis mellifera</i>	1583	38.8
Syrphid flies	<i>Eristalis tenax</i>	1594	39.1
	<i>Eupeodes corollae</i>	218	5.3
	<i>Sphaerophoria scripta</i>	198	4.9
	<i>Episyrphus balteatus</i>	35	0.9
	<i>Scaeva pyrastris</i>	29	0.7
	<i>Helophilus pendulus</i>	18	0.4
	<i>Syrphus</i> spp.	7	0.2
	Elves (<i>Meliscaeva</i> spp., <i>Meligramma</i> spp., <i>Melangyna</i> spp., <i>Fagisyrphus</i> spp., <i>Epistrophella</i> spp.)	1	0.0
	other Syrphidae	110	2.7
Solitary bees	<i>Andrena</i> spp. <i>Lasioglossum</i> spp.	92	2.3
Flies	Calypttrata	91	2.2
Bumblebees	<i>Bombus lapidarius</i>	18	0.4
	<i>B. pascuorum</i>	5	0.1
	<i>B. terrestris</i>	2	0.0
	<i>B. hypnorum</i>	1	0.0
	other <i>Bombus</i> spp.	4	0.1
Butterflies	<i>Pieris</i> spp.	10	0.2
	other Lepidoptera	6	0.1
Wasps	<i>Vespa</i> spp., <i>Polistes</i> spp.	16	0.4
Other insects	Unidentified	31	0.8
	Total	4077	



Overall pollinator visitation was affected by the feeding guild of the herbivores infesting the plants (Tables 3 and 4). The effect depended marginally on time point and was more apparent two weeks after flowering than one week after flowering (Tables 3 and 4). Two weeks after flowering, plants attacked by phloem feeders received fewer pollinator visits compared to undamaged plants, to plants attacked by chewers and to plants attacked by a mix of phloem feeders and chewers (i.e. Mixed) (Fig. 5). In addition, two weeks after flowering the overall pollinator visitation was affected by specific herbivores (Fig. 6). Plants attacked by the phloem feeders *L. erysimi* or *M. persicae* sub. *nicotianae* were visited by fewer pollinators than plants that did not receive herbivory, than plants that were attacked by *Athalia rosae*, by *M. brassicae*, by *Pieris brassicae*, or by a mix of one chewer and one phloem feeder (Fig. 6). The overall flower visitation by pollinators was marginally affected by the interaction between herbivore richness and feeding guild (Table 3). Overall flower visitation was not affected by specific herbivores (Fig. 7). Compared to control plants, the number of flowers visited by pollinators was not different on plants attacked by one or four species of phloem feeders (i.e. Phlo-1 or Phlo-4), by four species of chewers (i.e. Chew-4) or by two or four species of a mix of chewers and phloem feeders (i.e. Mix-2, Mix-4) (Fig. 8). In contrast, the number of flowers visited by pollinators was higher on plants attacked by one leaf-chewer species than on control plants (Fig. 8).

Plant phenotypic traits are affected by herbivore richness and functional traits

Herbivore communities introduced early in the season affected flowering time, but not the onset of flowering of *B. nigra* plants, and effects depended on herbivore identity rather than trait composition or richness of the herbivore community (Figs. 9 and 10). *M. persicae* sub. *nicotianae* induced shorter flowering compared to any other treatment (Fig. 10). Interestingly, none of the other phloem feeders, nor the combination of the four phloem feeders, reduced flowering time compared to control plants. On the contrary, plants that were attacked by *B. brassicae* flowered longer than plants attacked by the four chewers or than plants attacked by *P. brassicae* (Fig. 10).



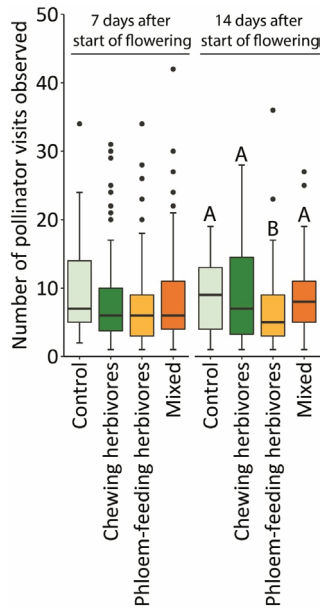


Figure 5. Number of pollinator visits observed on flowers of *Brassica nigra* plants treated with different herbivores grouped by feeding guild or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles. Observations lasted for 10 minutes and were made at two time points: between 7 and 9 days, and 14 and 16 days after plots had started flowering. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments within a time point based on adjusted Tukey's post hoc tests, small and capital letters were used for different time points.



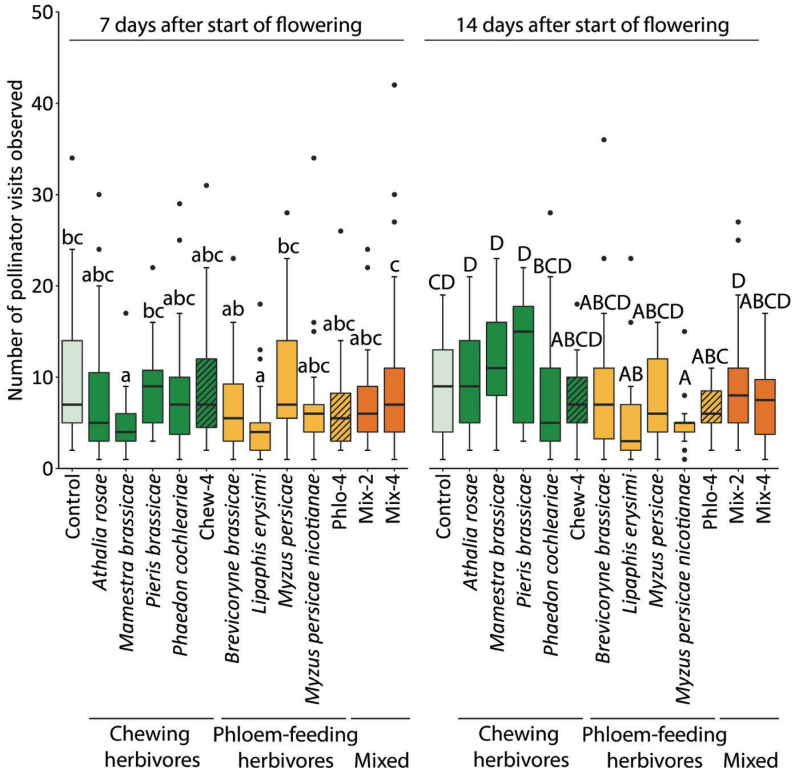


Figure 6. Number of pollinator visits observed on flowers of *Brassica nigra* plants treated with different herbivores or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles. Observations lasted for 10 minutes and were made at two time points: between 7 and 9 days, and 14 and 16 days after plots had started flowering. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments within a time point based on non-adjusted Tukey's post hoc tests, small and capital letters were used for different time points.



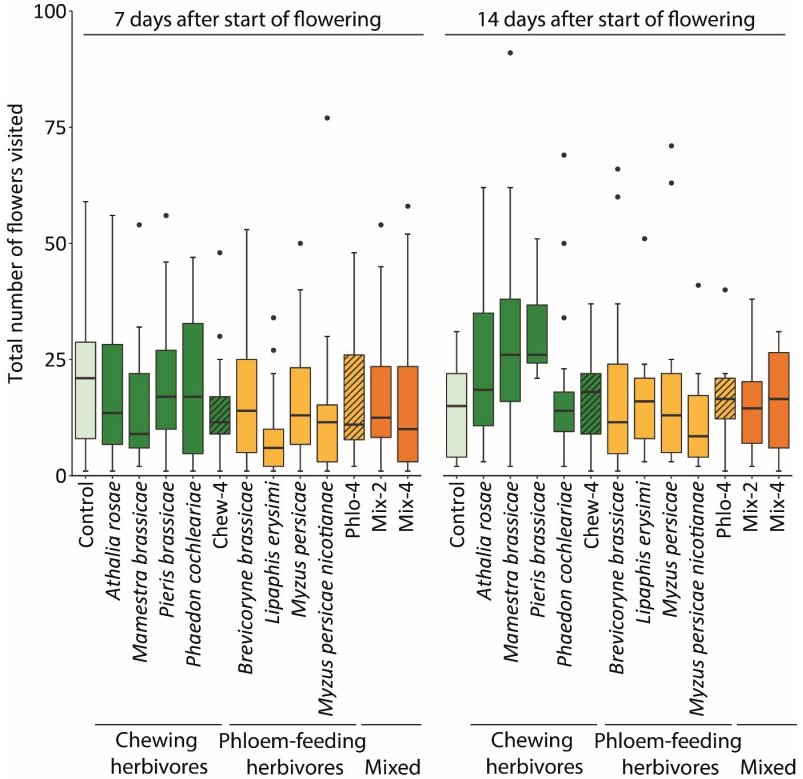


Figure 7. Number of flowers visited by all pollinators on *Brassica nigra* plants treated with different herbivores or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles. Observations lasted for 10 minutes and were made at two time points: between 7 and 9 days, and 14 and 16 days after plots had started flowering. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments within a time point based on non-adjusted Tukey's post hoc tests, small and capital letters were used for different time points.



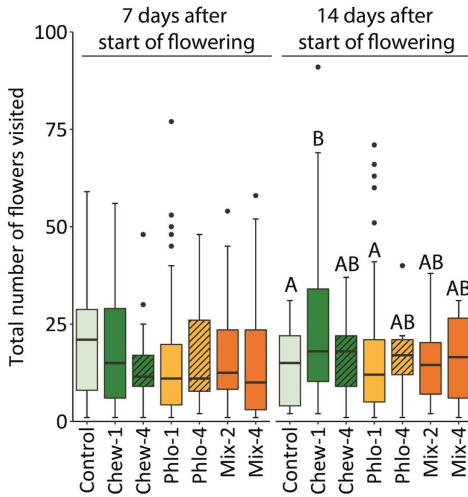


Figure 8. Number of flowers visited by all pollinators on *Brassica nigra* plants treated with different herbivores grouped by feeding guild and species richness or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles. Observations lasted for 10 minutes and were made at two time points: between 7 and 9 days, and 14 and 16 days after plots had started flowering. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments within a time point based on Tukey's post hoc tests, small and capital letters were used for different time points.



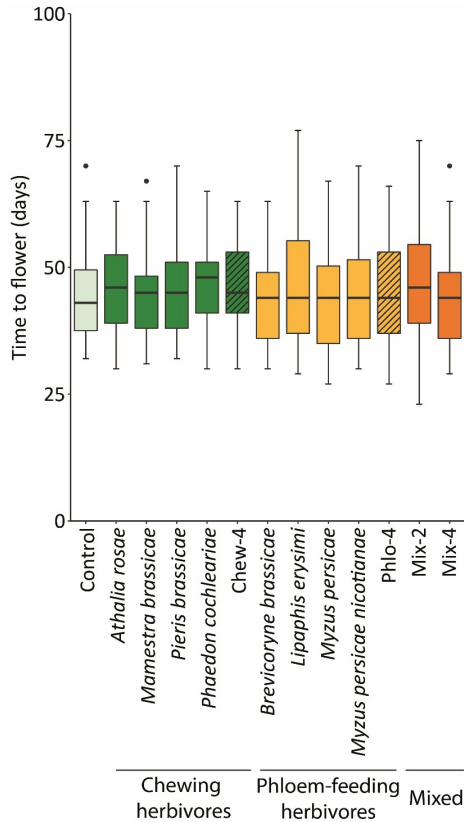


Figure 9. Time needed for *Brassica nigra* plants to start flowering after being treated with different herbivores or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles.



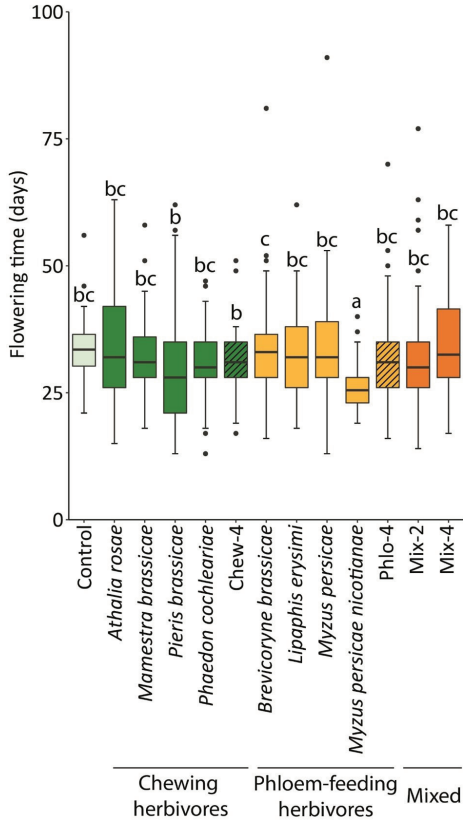


Figure 10. Flowering time of *Brassica nigra* plants after being treated with different herbivores or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments based on non-adjusted Tukey's post hoc tests.



Plant seed production

The number of seeds produced by *B. nigra* plants was not directly affected by herbivore species richness ($df = 3$, $\chi^2 = 6.34$, $P = 0.096$) nor by herbivore feeding guild ($df = 2$, $\chi^2 = 4.04$, $P = 0.133$). However, specific herbivore species reduced the number of seeds produced ($df = 12$, $\chi^2 = 21.48$, $P = 0.044$). Plants attacked by *A. rosae*, by *Myzus persicae* sub. *nicotianae*, and by the highest richness of chewers and phloem feeders (i.e. Chew-4 and Phlo-4 respectively) produced fewer seeds compared to plants that did not receive herbivory (Fig. 11). Seed weight was affected by herbivore species richness ($df = 3$, $\chi^2 = 11.40$, $P = 0.010$), herbivore feeding guild ($df = 2$, $\chi^2 = 6.89$, $P = 0.032$), and by their interaction ($df = 1$, $\chi^2 = 6.34$, $P = 0.041$) (Fig. 12). The richness of attackers only affected seed weight on mixed-induced plants. Plants attacked by the lowest richness of the mixture of phloem feeders and chewers (i.e. Mix-2) produced fewer seeds than plants attacked by the higher mixed species richness (i.e. Mix-4) (Fig. 12). The seed weight produced by Mix-2-induced plants was lower than plants that did not receive initial herbivore treatment or than plants that were induced by one species of phloem feeder (Phlo-1) (Fig. 12). In addition to trait composition and richness of the attacker community, specific herbivores affected the seed weight produced by *B. nigra* plants ($df = 12$, $\chi^2 = 44.09$, $P = 0.001$). *Athalia rosae*-treated and Mix-2-induced plants produced lighter seeds whereas *B. brassicae*-treated and Mix-4-induced plants produced heavier seeds (Fig. 13).

Direct and indirect effects of community members on plant fitness

Structural equation modelling (SEM) shows that the introduced herbivore communities directly and indirectly altered interactions between community members and that this change in interactions has consequences for plant fitness (Fig. 14). Plant performance parameters were strong indicators of plant reproductive success. Plants with more leaves produced more but lighter seeds (i.e. positive correlation of number of leaves with seed number but negative correlation with seed weight) (Fig. 14). Bigger plants (i.e. bigger



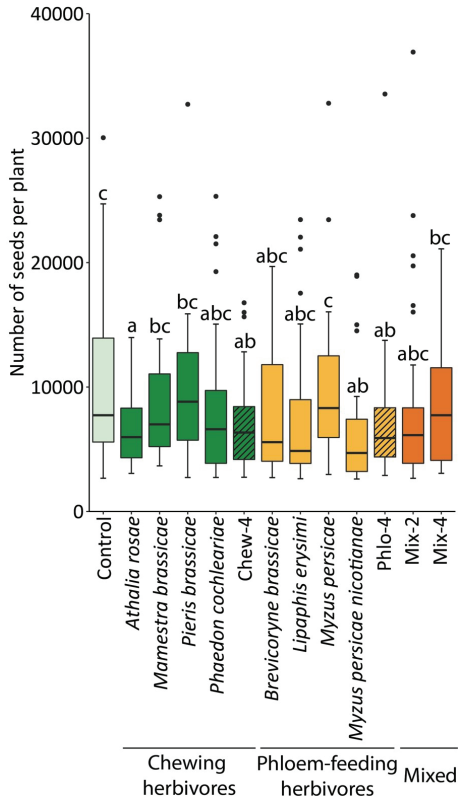


Figure 11. Number of seeds produced by *Brassica nigra* plants after being treated with different herbivores or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments based on non-adjusted Tukey's post hoc tests.



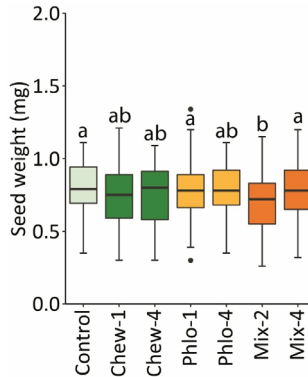


Figure 12. Weight of individual seeds produced by *Brassica nigra* plants after receiving different herbivore treatments grouped by species richness or feeding guild or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments based on adjusted Tukey's post hoc tests.

plant radius) produced heavier seeds (Fig. 14). Plants with a longer duration of flowering produced more and heavier seeds (Fig. 14). The introduced herbivore communities affected plant fitness frequently by altering plant performance traits. *Myzus persicae* was the only herbivore with a direct positive connection with seed weight (Fig. 14). *Myzus persicae*-induced and *B. brassicae*-induced plants produced heavier seeds explained by a reduction in the number of leaves of the plant (Fig. 14). In contrast, *A. rosae* and a mix of one phloem feeder and one chewer (i.e. Mix-2) had a negative effect on seed weight by reducing the duration of flowering (Fig. 14). The amount of leaf damage positively affected seed weight by increasing the duration of flowering (Fig. 14). Although the introduced herbivore communities affected abundance of *P. xylostella*, its parasitism rate and plant visitation by pollinators (Fig. 14), these effects were weakly connected to plant fitness parameters. The strongest connections were made through multiple interactions among organisms and were often via plant traits (Fig. 14). In itself, the abundance of *P. xylostella* did



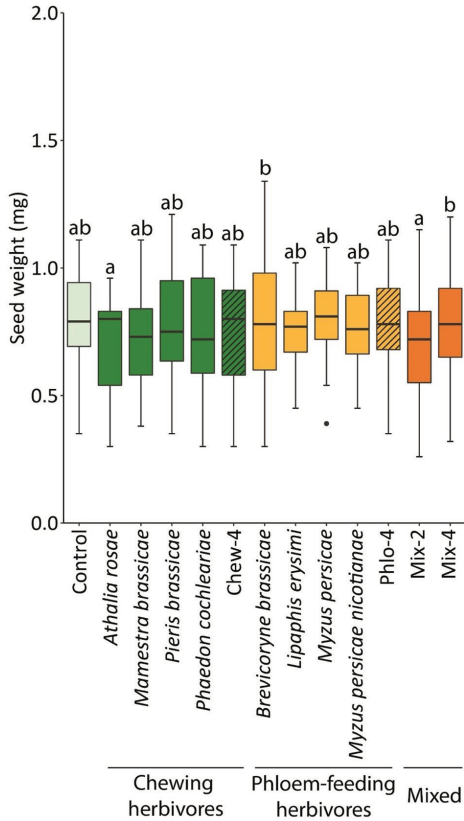


Figure 13. Weight of individual seeds produced by *Brassica nigra* plants after receiving the herbivore treatments or left untreated. Boxplots show median (line), first and third quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the first or above the third quartile) are represented by circles. Letters above bars indicate significant differences ($P \leq 0.05$) between herbivore treatments based on non-adjusted Tukey's post hoc tests



not correlate with plant fitness (number of seeds and seed weight). However, the parasitism rate of *P. xylostella* one week after induction negatively correlated with the number of seeds produced by the plants (Fig. 14). Pollinators were the strongest predictors for plant fitness (Fig. 14). The number of pollinators visiting the plant had a positive effect on the number of seeds produced by increasing the number of flowers visited per plant (Fig. 14). Flowering time had a negative effect on the numbers of seeds produced because it reduced the numbers of flowers visited by pollinators (Fig. 14). By interaction with pollinators, the abundance of *P. xylostella* and its parasitism rate affected plant fitness. Abundance of *P. xylostella* had an indirect positive effect on the number of seeds produced by increasing the number of flowers visited by pollinators (Fig. 14). The parasitism rate of *P. xylostella* positively correlated with the number of flower visitors, that enhanced the number of flowers visited and positively correlated with seed set. These interactions were differentially influenced by the composition of the herbivore community introduced early in the season. Introduction of a species rich phloem feeder community (Phlo-4) negatively correlated with plant damage, that corresponded with higher parasitism rates, cascading to increased number of flower visitors and visitations that enhanced seed number (Fig. 14). Thus, introduction of the phloem feeder community eventually resulted in lower plant fitness (see also Fig. 11). A similar negative effect on seed number could be deduced for the specific phloem feeder *L. erysimi* that reduced the number of flowers visited by pollinators (Fig. 14). Also, the species-rich leaf chewer community (Chew-4) reduced the number of flowers visited by pollinators that positively correlated with seed number, thus yielding overall reduced fitness. A mix of herbivores (Mix-2, Mix-4), three out of four leaf chewer species (*A. rosae*, *P. cochleariae*, *P. brassicae*) and three out of four phloem feeder species (*Myzus persicae*, *Myzus persicae nicotianae*, *B. brassicae*) positively affected abundance of *P. xylostella* that in turn positively correlated with the number of flowers visited by pollinators, which resulted in increased seed set.



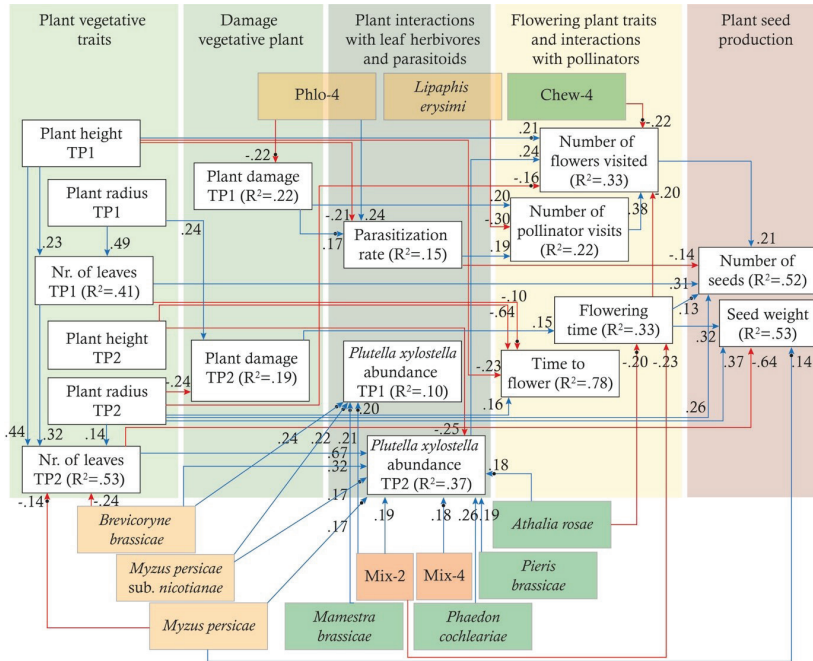


Figure 14. Structural Equation Modelling showing variables directly affected by the herbivore treatments, Blue arrows represent positive links whereas, red negative. A dot in front of the arrows represents correlations where $0.05 < P < 0.10$. TP1 and TP2 refer to measurements made at the first and second time point, respectively. Chew-4, and Phlo-4 are plants that received four species of chewers and phloem feeders, respectively. Mix-2 and Mix-4 are plants that respectively received four or two species of a mix of chewers and phloem feeders.

DISCUSSION

Herbivore richness, its combination of functional traits and specific herbivore species affected the abundance of *P. xylostella*, the parasitism rate of *P. xylostella* and pollinator visitation to *B. nigra* plants. The composition of the initial herbivore community affected plant fitness predominantly through its effects on plant performance such as growth, flowering time, and amount of leaf damage that strongly correlated with number and weight of seeds produced. In addition, species-rich communities of a single feeding guild (either only leaf chewers



or only phloem feeders) negatively affected plant fitness indirectly through a chain of interactions involving reduced pollinator visitation rates that positively correlated with seed number. In contrast, plants induced with a mix of phloem feeders and leaf chewers, as well as plants induced by individual herbivore species, promoted the presence of *P. xylostella* that corresponded with enhanced flower visitation and increased seed production. These indirect interactions that resulted in positive effects on seed production did in most cases not compensate for the negative effect of herbivores on fitness via effects on plant performance.

Herbivore richness, herbivore functional traits and herbivore identity affected the interaction network of *B. nigra*. In general, *P. xylostella* was more abundant on plants that received herbivory early in the season regardless of the richness and the functional traits, compared with control plants. Likewise, in a field study *P. xylostella* was more abundant on *Brassica oleracea* plants attacked by *Pieris rapae*, compared with undamaged plants (Poelman *et al.*, 2008). In our study, not only *P. xylostella* was affected by herbivory, but also its parasitoids. The parasitism rate of *P. xylostella* larvae was affected by specific herbivores attacking the plant, and by their feeding guild. Plants attacked by a mix of two chewers and two phloem feeders (i.e. Mix-4) had higher rates of parasitism than parasitism rates on control plants. A recent study showed that compared to the performance on undamaged plants, *P. xylostella* grew less on plants induced by chewers, while on mixed-induced plants the performance was not reduced (Fernández de Bobadilla *et al.*, 2021). Parasitoids may be more attracted to mixed-induced plants because of the higher detectability and the association of the mixed-induced volatiles with a higher quality of the host (Aartsma *et al.*, 2019). Additionally, in a choice assay in the greenhouse, *P. xylostella* laid fewer eggs on plants attacked by a mix of two chewers and two phloem feeders than on plants attacked by phloem feeders. The reduced preference of *P. xylostella* towards the plants that in the field received the highest parasitism may be indicative of an adaptation of *P. xylostella* to avoid herbivore communities that predict high risk of parasitism.



In our study, *P. xylostella* abundance did not have direct effects on plant fitness. Thus herbivore-herbivore interactions between the species we studied did not explain fitness. However, *P. xylostella* abundance connected with an increase in the number of flowers visited by pollinators, which was positively correlated with plant fitness. This suggests that interactions from early-season herbivory to a later arriving herbivore that affects flower visitation by pollinators imposes selection on plant traits (Mertens *et al.*, 2021; Rusman *et al.* 2019a). Similarly, simulated florivory in *Impatiens capensis* plants, reduced pollinator and pollen thief visits, and the alterations in interactions affected plant fitness (Soper Gorden & Adler, 2016, 2018). Plants attacked by the phloem feeder *L. erysimi* and by the four chewers simultaneously (i.e. Chew-4) received fewer visits by pollinators. Likewise, a recent study shows that herbivory by aphids caused nectar shortage which reduced pollination visitation and led to a reduction in plant fitness (Devegili & Chalcoff, 2020). The herbivore-induced effects on plant interactions with herbivores, parasitoids and pollinators could be attributed to a change in important cues for insect foraging, such as the volatile blend or visual cues (Kessler & Chautá, 2020; Moreira *et al.*, 2019; Rusman *et al.*, 2019b).

Plant fitness was affected by the richness of herbivores attacking the plant, by their feeding guild, and by their interaction. Attack by the highest richness of chewers and phloem feeders (i.e. Chew-4 and Phlo-4 respectively) as well as by *A. rosae*, or by *M. persicae* sub. *nicotianae* negatively affected plant fitness in terms of seed numbers. The negative effect on plant fitness was connected with a reduction in pollinator visitation. Additionally, when feeding individually, the two most common aphids attacking *B. nigra* (i.e. *M. persicae* and *B. brassicae*) (Mertens *et al.*, 2021) had a positive effect on seed weight by inducing a reduction in the number of leaves. The negative correlation between number of leaves and seed production could be explained by the plant reallocating the energy used for leaves to other parts, such as the roots, increasing the plant nutrient uptake (Ruan *et al.*, 2013). Additionally, a reduction in numbers of leaves could make the plant less attractive to herbivores not monitored in this study that have strong fitness consequences. However, we did not find any correlation between



seed weight and leaf damage. Therefore, the herbivore reducing plant fitness on plants with fewer leaves should be a species that does not cause notable leaf damage to the plant at the time measured (e.g. a root- or phloem-feeding herbivore). *Delia radicum* larvae feed on Brassicaceae roots, causing high fitness costs being unnoticed for weeks (Soler *et al.*, 2005, 2007). Alternatively, the estimated leaf damage of a full plant might not be a good predictor of plant fitness (de Vries *et al.* 2017, 2018, 2019). In contrast to the positive effect of some individual phloem feeder species on plant fitness, when phloem feeders attacked the plant in combination with chewers (i.e. Mix) the collective effect on plant fitness was negative. Induction of plants with only leaf chewers also reduced plant fitness. Plants attacked by the chewer *A. rosae* and a by mix of one chewer and one phloem feeder (i.e. Mix-2) produced lighter seeds through reduced flowering time. The reduction in seed weight linked to shorter flowering is potentially explained by a reduction in pollinator visitation or by lower quality pollination of individual flowers.

We show that herbivore richness, functional trait diversity and herbivore identity affect plant fitness and plant interactions with *P. xylostella*, parasitoids and pollinators. Herbivory strongly affected plant fitness through reducing plant performance and flowering time. Indirect interactions between subsequent herbivores, parasitoids and pollinators affected plant fitness mediated by plant traits. All of these interactions involved effects of inducing herbivores on pollinators that closely connected with the total number of seeds produced by a plant. Under climate change and insect decline, where herbivore communities are predicted to be poorer in species richness, we may expect effects on plant trait evolution. One of the groups that is already suffering a worrying decline is pollinators (Althaus *et al.*, 2021). If the pollinator decline situation continues, pollinator limitation will likely magnify the fitness consequences of indirect interactions observed in our study, as all the routes affecting plant fitness connected to pollinators. Additionally, the natural biological control of herbivores by parasitoids is expected to be disrupted due to climate change (Chidawanyika *et al.*, 2019). Under climate change, predictions pose that plants



will suffer more stress and the extensive effects of herbivore richness, functional trait and identity on the interaction network will likely add to this impact on plant fitness (Wagner *et al.*, 2021).

Our study has uncovered the tip of the iceberg of how herbivore richness, functional trait and identity alters direct and indirect interactions with other insects and has fitness effects on plants. Our work allows us to predict that the current situation of insect decline and climate change will have dramatic consequences on the structure of the community interacting with a plant. However, due to the complexity and high connectivity of the interactions network it is very difficult to make predictions on directions of effects for plant trait selection.

ACKNOWLEDGEMENTS The authors thank Sean Zending for helping in data collection of the plant flowering traits and pollinator visitation. We also thank Karin Winkler for her kind help with the design of the device used for the parasitism of *P. xylostella* experiment. We thank the staff of Unifarm for setting up and maintaining the experimental fields.



SUPPLEMENTARY INFORMATION

Materials and methods from experiments studying the effect of herbivore richness and guild on the oviposition preference of *Plutella xylostella* on *Brassica nigra* plants

Plants and insects

3-4 weeks old black mustard, *Brassica nigra* (Brassicales: Brassicaceae) were used for the experiments. The plants were grown and maintained in a greenhouse at 22 ± 2 °C, 60-70 % RH and 16:8 h L:D photo regime. Insects used as inducers were the same species and reared in the same conditions as the ones used the field experiment (see main text).

*Plant induction and *Plutella xylostella* oviposition preference*

Four leaves of each plant were induced with the herbivore treatments, which were the same treatments that were used in the field study (Con-0, Phlo-1, Phlo-4, Chew-1, Chew-4, Mix-2, Mix-4). Each of the four leaves received the total number of herbivores applied on the whole plant in the field. After herbivore infestation, each leaf was enclosed in a mesh bag, to prevent the movement of the herbivores to different leaves. The induction lasted for five days. Con-0 plants functioned as control, these plants did not receive any inducing herbivore, but were treated in a similar way as plants receiving herbivores. Plants that received the different treatments were placed in the greenhouse in a randomized block design.

We prepared plastic oviposition cages, which had as base a large Petri dish (145/20 mm) on which plastic sheets (210x297 mm) were installed to create the walls of the cages (as described in Poelman *et al.*, 2008) (Fig S1). Two 5 ml glass vials were glued to the bottom of the oviposition cages and were filled with water. Right before the oviposition preference assays, one leaf from each of the two plants to test was detached from the plant with scissors, and the leaf petiole was immediately submerged in one of the two vials filled with water, to avoid dehydration, and the herbivores were removed with a brush. To



avoid differences in oviposition preference caused by leaf age, pairs of leaves of similar age were chosen for the two-choice set-up. The leaves were placed in a way that prevented them from touching each other and were randomly assigned to one of two positions in the plastic cage (Fig S1).

The oviposition preference of the diamondback moth, *Plutella xylostella* L. (Lepidoptera: Plutellidae) between leaves of plants that received different treatments was studied. Oviposition preference of *P. xylostella* moths is mostly driven by post alighting gustatory perception of non-volatile glucosinolates, leaf surface waxes, chemosensory stimulation and abiotic factors (Justus & Mitchell, 1996; Zhang *et al.*, 2012). One female *P. xylostella* moth was released in each oviposition cage together with one adult male, to ensure mating. The exact age of the moths was unknown, and younger and older butterflies were mixed to randomize our design. Since it is known that *P. xylostella* prefers to oviposit at dusk, the oviposition preference was measured twenty-four hours after the release of the couples. This was done by carefully inspecting and counting the number of eggs found on each leaf. The number of eggs present on the walls of the oviposition cage was also recorded. The leaves of each choice set-up and the *P. xylostella* moths were used only once. Each oviposition cage was considered as one replicate.

Oviposition preference comparisons

To test if the oviposition preference of *P. xylostella* moths is affected by the herbivore treatments, we conducted two main experiments, performed one after each other in the same greenhouse compartment. The first experiment was designed to evaluate the effect of herbivory and of herbivore guild on the oviposition preference of *P. xylostella*. To test the effect of herbivory, *P. xylostella* moths were allowed to choose oviposition site between a leaf from an undamaged plant (i.e. Con-0) or from a plant induced with the highest richness of phloem feeders, leaf chewers or a mix of both (i.e. Phlo-4, Chew-4 or Mix-4). To test the effect of guild of the attacker, the moths were allowed to choose oviposition site between leaves from the highest herbivore richness of phloem



feeders, chewers or a mix (Table S1). The experiment was divided in four blocks over time, performed one after each other. Each choice was repeated 12 times in each of the blocks, making a total of 48 replicates per treatment comparison.

In a second experiment we studied the effect of herbivore richness within feeding guild on the oviposition preference of *P. xylostella*. The moths were allowed to choose between leaves induced with the lowest or highest richness of herbivores within each guild (Table S2). This experiment was also divided in four blocks over time, performed one after each other. For the effect of phloem-feeder and chewer richness, each combination was repeated eight times in each of the blocks, making a total of 32 replicates per treatment comparison. For the mixed richness, two species of Mix-2 were compared with the same two species of Mix-2 plus two more species. We selected treatments, so that each combination of one phloem feeder and one chewer species of Mix-2 was tested against three different combinations of Mix-4, making a total of 48 different comparisons (Table S2). Due to the high number of choice combinations, and space and time limitations, each comparison was repeated only once in each of the four blocks.

Statistical analysis

In some cases, the moths did not lay any egg and these replicates were not included in the analysis (Table S3). In other cases, *P. xylostella* laid most eggs on the walls of the oviposition cage (Table S3). When the percentage of eggs found on the walls of the cage exceeded 50%, we excluded the replicate from further analysis (Table S3). Oviposition preference data was analysed using two-sided paired t-tests if normally distributed, or by Wilcoxon signed-rank tests in case the data did not follow a normal distribution. The normality was visually examined by making histograms of the difference of the eggs that were laid in the 1st choice leaf and the 2nd choice leaf in each dual oviposition preference assay (in each oviposition cage). All the statistical analyses were performed using RStudio version 1.1.463.



Results from experiments studying the effect of herbivore richness and guild on the oviposition preference of *Plutella xylostella* on *Brassica nigra* plants

The oviposition preference of *P. xylostella* was not affected by herbivory. The preference of *P. xylostella* did not differ between undamaged leaves and leaves that were induced with the maximum richness of phloem feeders, chewers and a mix of both (Con-0 vs Phlo-4: $P = 0.1935$; Con-0 vs Chew-4: $P = 0.8736$; paired t-test; Con-0 vs Mix-4: $P = 0.1842$; Wilcoxon signed rank test Fig. S2A-C)). *P. xylostella* female moths also did not discriminate between leaves induced with highest richness of phloem feeders and chewers, nor between leaves induced with the highest richness of chewers and mix (Phlo-4 vs Chew-4: $P = 0.87$, paired t-test; Chew-4 vs Mix-4: $P = 0.34$; Wilcoxon signed rank test; Fig. S2 D, E). In contrast, the moths preferred to lay eggs on leaves induced with the highest richness of phloem feeders compared to leaves induced with the highest richness of the mix (Phlo-4 - Mix-4: $P < 0.001$; Wilcoxon signed rank test, Fig. S2F).

The richness of phloem feeders did not affect the oviposition preference of *P. xylostella* and the number of eggs laid by the moths on leaves induced by one species or by four species of phloem feeders was not different (Phlo-1 vs Phlo-4: $P = 0.57$, paired t-test; Fig. S3). No significant differences were found in the oviposition preference on leaves induced with *Brevicoryne brassicae*, *Lipaphis erysimi*, *Myzus persicae* and *Myzus persicae* sub. *nicotianae* compared with leaves induced with all phloem-feeding species simultaneously (*B. brassicae* vs Phlo-4: Wilcoxon signed rank test $P = 0.72$, *L. erysimi* vs Phlo-4: Wilcoxon signed rank test $P = 0.22$; *M. persicae* vs Phlo-4: Wilcoxon signed rank test $P = 0.17$; *M. persicae* sub. *nicotianae* vs Phlo-4: paired t-test $P = 0.10$; Fig. S3A-D). In line with the lack of phloem-feeder richness on the oviposition preference of *P. xylostella*, the moths did not discriminate between leaves induced with one or with four chewer species (Chew-1 - Chew-4: $P = 0.72$, paired t-test). However, some specific herbivores affected *P. xylostella* preference. Moths laid more eggs on *Phaedon cochleariae*- induced leaves



than on the maximum chewer diversity (*P. cochleariae* vs Chew-4: Wilcoxon signed rank test $P < 0.05$; Fig. S4C), while they preferred to oviposit on leaves induced by the highest chewer richness than on *Mamestra brassicae*-induced leaves (*M. brassicae* vs Chew-4: Wilcoxon signed rank test $P < 0.01$; Fig. S4B). In contrast, *P. xylostella* did not discriminate between leaves induced with the highest richness of chewers and *Pieris brassicae*-induced or *Athalia rosae*-induced leaves (*P. brassicae* - Chew-4: paired t-test $P = 0.46$; *A. rosae* - Chew-4: Wilcoxon signed rank test $P = 0.54$; Fig. S4 A,D). The oviposition preference of *P. xylostella* was also not affected by the richness of herbivores on a mix of phloem feeders and chewers (Mix-2 vs Mix-4: paired t-test $P = 0.58$; Fig. S5).

Figures from experiments studying the effect of herbivore richness and guild on the oviposition preference of *Plutella xylostella* on *Brassica nigra* plants

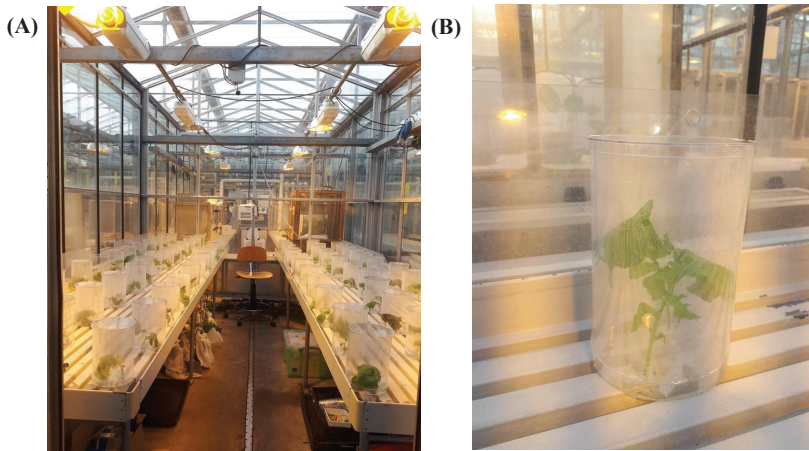


Figure S1: (A) Greenhouse compartment with the cages in which we tested the oviposition preference of *Plutella xylostella* in one block. (B) Closer look of one oviposition cage, with two leaves coming from two different induction treatments with their petioles in the two glass vials.



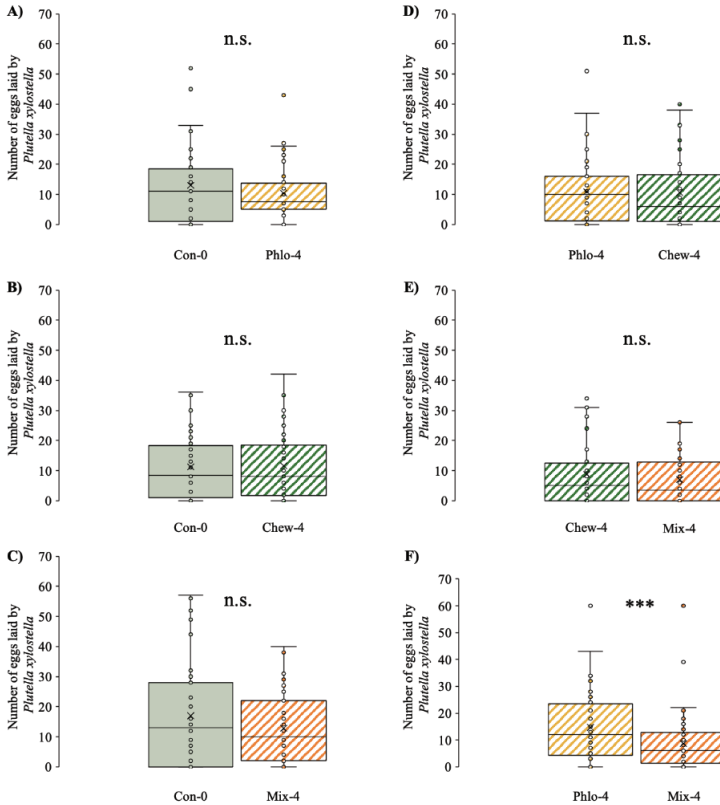


Figure S2. Number of eggs laid by *Plutella xylostella* moths on each *Brassica nigra* leaf of the two-choice assay between **A)** Untreated plants (Con-0) and plants treated with the highest species richness of phloem feeders (Phlo-4). **B)** Untreated plants (Con-0) and plants treated with the highest species richness of chewers (Chew-4). **C)** Untreated plants (Con-0) and plants treated with the highest species richness of a mix of phloem feeders and chewers (Mix-4). **D)** Plants treated with the highest species richness of phloem feeders (Phlo-4) and plants treated with the highest species richness of chewers (Chew-4). **E)** Plants treated with the highest species richness of chewers (Chew-4) and plants treated with the highest species richness of a mix of phloem feeders and chewers (Mix-4). **F)** Plants treated with the highest species richness of phloem feeders (Phlo-4) and plants treated with the highest species richness of a mix of phloem feeders and chewers (Mix-4). Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. Asterisks above the boxplots show significant differences (***P < 0.001; Wilcoxon signed rank test). n.s.=non-significant differences (P > 0.05; Wilcoxon signed rank test).



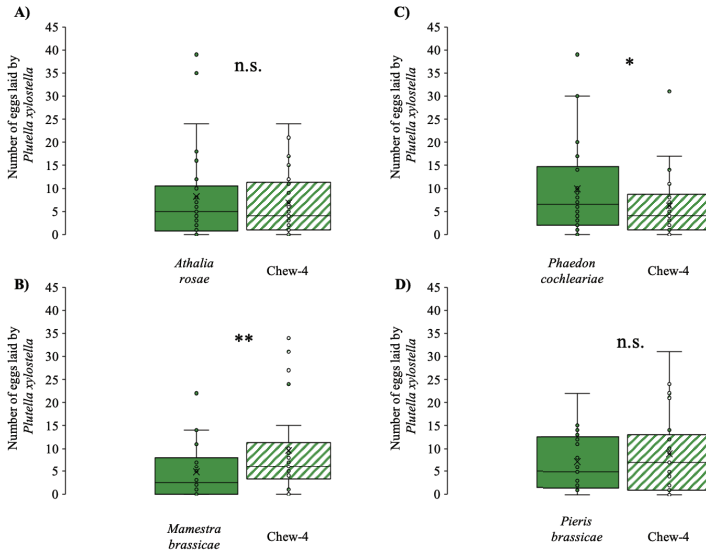


Figure S3. Number of eggs laid by *Plutella xylostella* moths on each *Brassica nigra* leaf of the two-choice assay between **A**) Plants treated with the chewer *Athalia rosae* and plants treated with the highest species richness of chewers (Chew-4). **B**) Plants treated with the chewer *Mamestra brassicae* and plants treated with the highest species richness of chewers (Chew-4). **C**) Plants treated with the chewer *Phaedon cochleariae* and plants treated with the highest species richness of chewers (Chew-4). **D**) Plants treated with the chewer *Pieris brassicae* and plants treated with the highest species richness of chewers (Chew-4). Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. Asterisks above the boxplots show significant differences (* $P < 0.05$; ** $P < 0.01$; Wilcoxon signed rank test). n.s.=non-significant differences ($P > 0.05$; Wilcoxon signed rank test).



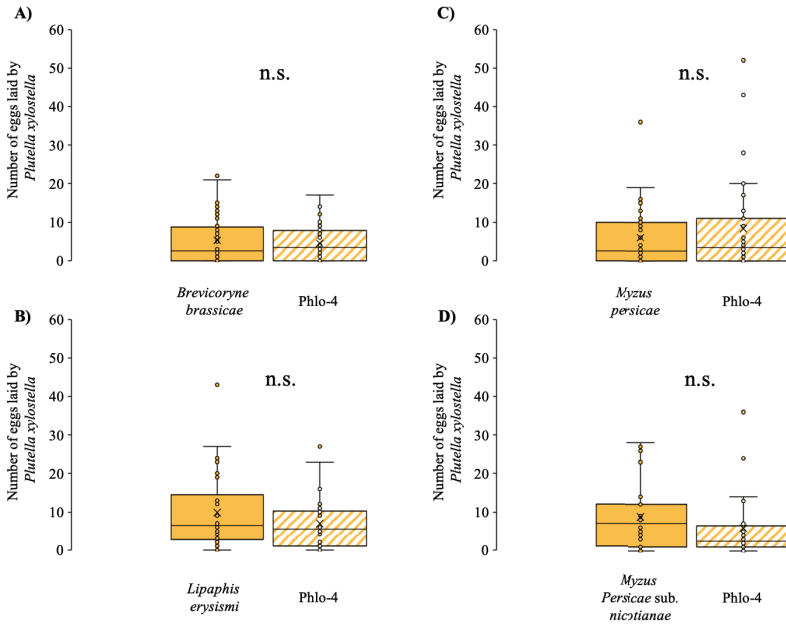


Figure S4. Number of eggs laid by *Plutella xylostella* moths on each *Brassica nigra* leaf of the two-choice assay between **A)** Plants treated with the phloem feeder *Brevicoryne brassicae* and plants treated with the highest species richness of phloem feeders (Phlo-4). **B)** Plants treated with the phloem feeder *Lipaphis erysimi* and plants treated with the highest species richness of phloem feeders (Phlo-4). **C)** Plants treated with the phloem feeder *Myzus persicae* and plants treated with the highest species richness of phloem feeders (Phlo-4). **D)** Plants treated with the phloem feeder *M. persicae sub. nicotianae* and plants treated with the highest species richness of phloem feeders (Phlo-4). Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. n.s.=non-significant differences ($P > 0.05$; Wilcoxon signed rank test).



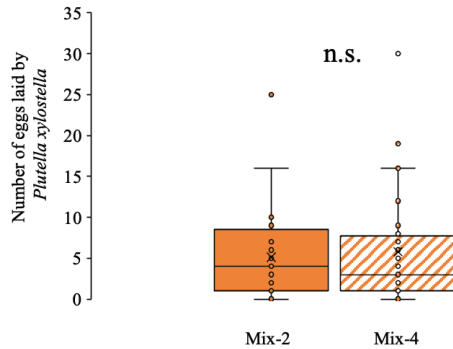


Figure S5. Number of eggs laid by *Plutella xylostella* moths on each *Brassica nigra* leaf of the two-choice assay between plants treated with the two or with four species of a mix of phloem feeders and chewers (Mix-2 or Mix-4). Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. Asterisks above the boxplots show significant differences. n.s.=non-significant differences ($P > 0.05$; Wilcoxon signed rank test).

Table S1. Herbivore combinations included in the two-choice oviposition experiment 1. The experiment was divided in four blocks over time. Each comparison was repeated 12 times per block, making a total of 48 replicates per choice.

Leaf 1	Leaf 2
Con-0	Phlo-4
Con-0	Chew-4
Con-0	Mix-4
Phlo-4	Chew-4
Phlo-4	Mix-4
Chew-4	Mix-4



Table S2. Herbivore combinations included in the two-choice oviposition experiment 2. The experiment was divided in four blocks over time. The phloem feeder and chewer richness comparisons were repeated eight times per block, making a total of 32 replicates per choice. Each of the 48 Mix-2 Mix-4 comparisons was repeated only once. Phloem feeding aphids *Brevicoryne brassicae* (*Bb*), *Lipaphis erysimi* (*Le*), *Myzus persicae* (*Mp*), *Myzus persicae* sub. *nicotianae* (*Mpn*). Chewers: *Athalia rosae* (*Ar*), *Mamestra brassicae* (*Mb*), *Phaedon cochleariae* (*Pc*), *Pieris brassicae* (*Pb*).

Leaf 1	Leaf 2
Phlo-1	Phlo-4
<i>Bb</i>	
<i>Mp</i>	<i>Bb+Mp+Mpn+Le</i>
<i>Mpn</i>	
<i>Le</i>	
Chew-1	Chew-4
<i>Ar</i>	
<i>Pb</i>	<i>Ar+Pb+Pb+Mb</i>
<i>Pc</i>	
<i>Mb</i>	
Mix-2	Mix-4
<i>BbMb</i>	
<i>BbAr</i>	<i>BbMp MbAr</i>
<i>MpMb</i>	
<i>MpAr</i>	
<i>BbPb</i>	
<i>BbPc</i>	<i>BbMp PbPc</i>
<i>MpPb</i>	
<i>MpPc</i>	
<i>BbMb</i>	
<i>BbPc</i>	<i>BbMpn MbPc</i>
<i>MpnMb</i>	
<i>MpnPc</i>	



Table S2. Continuation.

Leaf 1	Leaf 2
<i>BbAr</i>	
<i>BbPb</i>	<i>BbMpn ArPb</i>
<i>MpnPb</i>	
<i>MpnAr</i>	
<i>BbMb</i>	
<i>BbPb</i>	<i>BbLe MbPb</i>
<i>LeMb</i>	
<i>LePb</i>	
<i>BbAr</i>	
<i>BbPc</i>	<i>BbLe ArPc</i>
<i>LeAr</i>	
<i>LePc</i>	
<i>MpMb</i>	
<i>MpPb</i>	<i>MpMpn MbPb</i>
<i>MpnMb</i>	
<i>MpnPb</i>	
<i>MpAr</i>	
<i>MpPc</i>	<i>MpMpn ArPc</i>
<i>MpnAr</i>	
<i>MpnPc</i>	
<i>MpMb</i>	
<i>MpPc</i>	<i>MpLe MbPc</i>
<i>LeMb</i>	
<i>LePc</i>	



Table S3. Overview of replicates included for the oviposition experiments. “No response” indicates the number of set-ups where the moths did not lay any egg. The “No choice” indicates the set-ups where the moths laid more than 50% of the eggs on the oviposition cages instead than on the two choice leaves. The “No response” and “No choice” columns were excluded from the data analysis. Phloem feeding aphids *Bb*= *Brevicoryne brassicae*, *Le*= *Lipaphis erysimi*, *Mp*= *Myzus persicae*, *Mpn*= *Myzus persicae* sub. *nicotianae*, *Ar*= *Athalia rosae*, *Mb*= *Mamestra brassicae*, *Pb*= *Pieris brassicae*, *Pc*= *Phaedon cochleariae*.

Comparisons	Replicates tested	“No response”	“No choice”	Replicates included in the analysis
Con-0 vs Phlo-4	48	9	0	39
Con-0 vs Chew-4	48	6	1	41
Con-0 vs Mix-4	48	10	0	38
Phlo-4 vs Chew-4	48	7	1	40
Phlo-4 vs Mix-4	48	7	2	39
Chew-4 vs Mix-4	48	14	1	33
Phlo-1 vs Phlo-4/Total	128	26	3	99
<i>Bb</i> vs Phlo-4	32	6	0	26
<i>Le</i> vs Phlo-4	32	6	0	26
<i>Mp</i> vs Phlo-4	32	6	3	23
<i>Mpn</i> vs Phlo-4	32	8	0	24
Chew-1- Chew-4/Total	128	36	5	87
<i>Ar</i> vs Chew-4	32	6	2	24
<i>Mb</i> vs Chew-4	32	10	2	20
<i>Pb</i> vs Chew-4	32	11	0	21
<i>Pc</i> vs Chew-4	32	9	1	22
Mix-2 vs Mix-4	48	20	0	28





CHAPTER 6
**Plasticity in induced resistance to sequential
attack by multiple herbivores in *Brassica nigra***

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Under review at *Oecologia*

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.

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

Species (Abbreviation)	Picture	Order	Feeding guild
<i>Athalia rosae</i> (<i>Ar</i>)		Hymenoptera	Leaf chewer
<i>Plutella xylostella</i> (<i>Px</i>)		Lepidoptera	Leaf chewer
<i>Brevicoryne brassicae</i> (<i>Bb</i>)		Hemiptera	Phloem feeder
<i>Myzus persicae</i> (<i>Mp</i>)		Hemiptera	Phloem feeder

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).



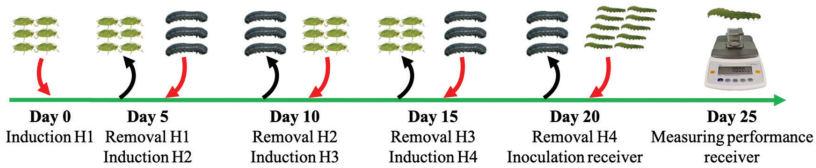


















































Figure 1. Experimental timeline depicting the herbivore sequences. On Day 0 each *Brassica nigra* plant was induced with the first set of herbivores (H1) which consisted of either three chewers or six aphids. After five days all the herbivores were removed from the plant using a brush, and the second 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 of plant resistance.

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 °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).

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*.

Treatment	H1	H2	H3	H4
Ctrl	-	-	-	-
Px-Px-Px-Px				
Ar-Ar-Ar-Ar				
Bb-Bb-Bb-Bb				
Mp-Mp-Mp-Mp				
Bb-Bb-Px-Px				
Bb-Px-Bb-Px				
Mp-Mp-Ar-Ar				
Mp-Ar-Mp-Ar				
Px-Px-Bb-Bb				
Px-Bb-Px-Bb				
Ar-Ar-Mp-Mp				
Ar-Mp-Ar-Mp				



Statistical analysis

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* ($F_{1,33} = 3$, $P = 0.04$). 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,



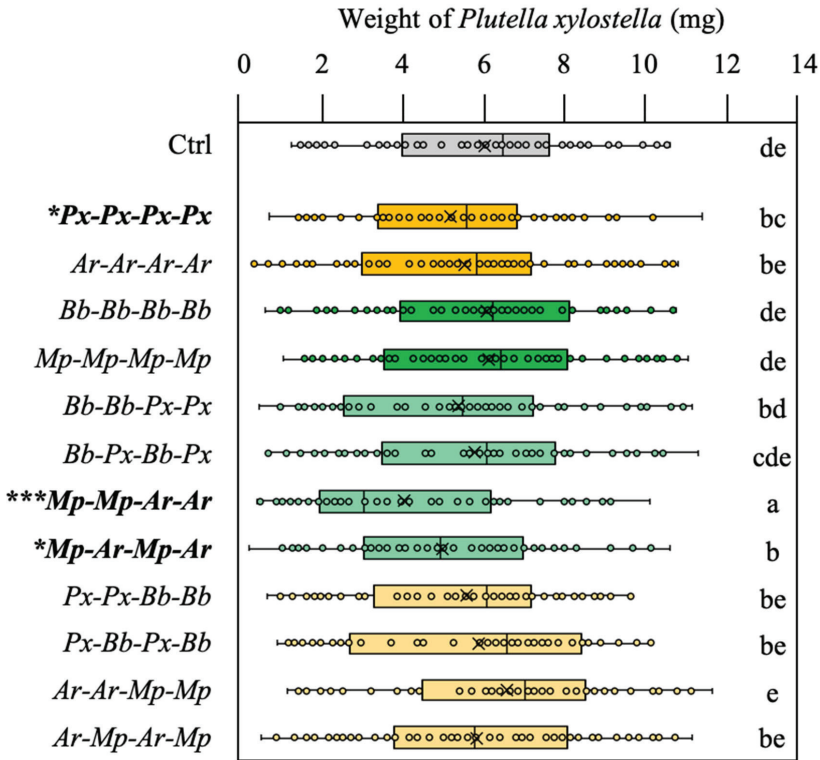


Figure 2. Weight (mg) of *Plutella xylostella* larvae after feeding for five days from *Brassica nigra* plants previously attacked by sequences of four herbivore events (N= 18). Darker boxplots indicate herbivore sequences that received four times the same herbivore. Yellow or green bars indicate treatments 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).



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: $F_{1,23}=-8.12$, $P<0.001$; 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).

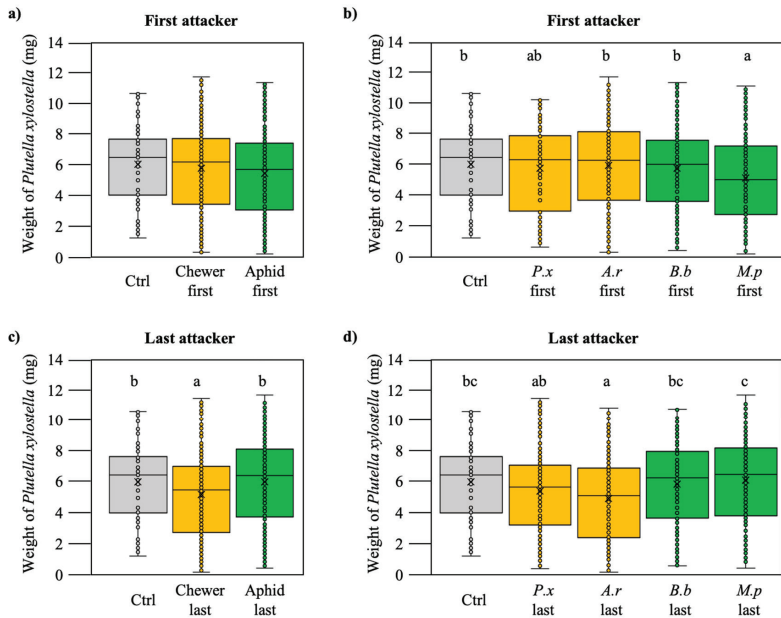


Figure 3. Effect of first or last attacker on resistance to *Plutella xylostella*. Weight (mg) of *P. xylostella* larvae after feeding as fifth herbivore from *Brassica nigra* plants that were previously attacked by four sets of herbivores where: **a)** the first attacker was either a chewer, an aphid 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).



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.*



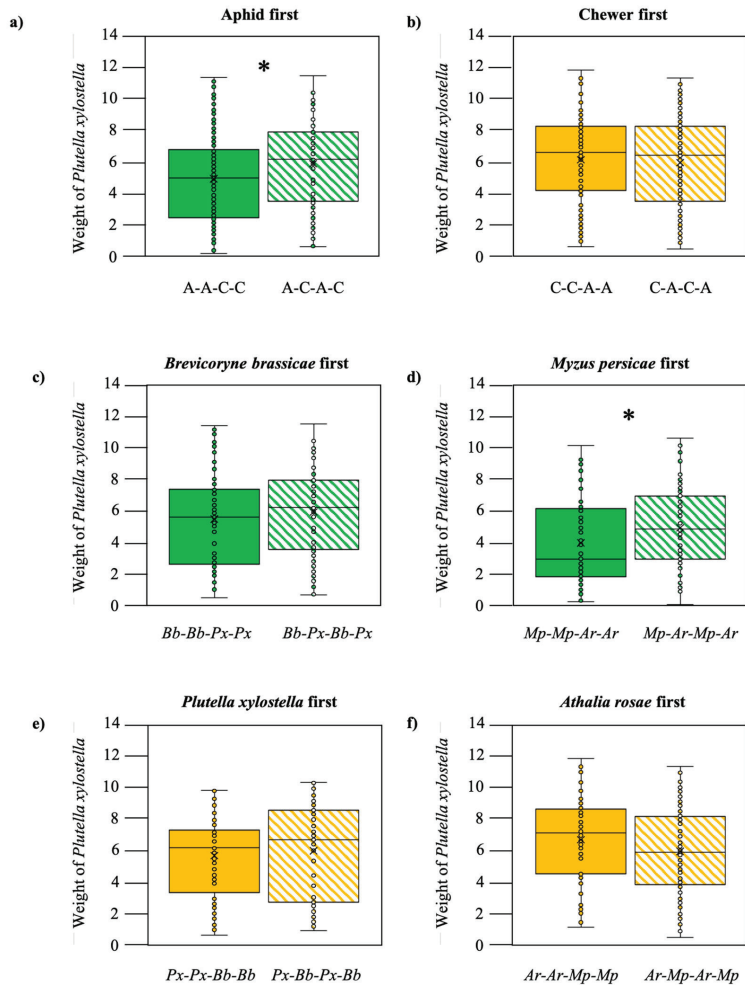


Figure 4. Weight (mg) of *Plutella xylostella* larvae after feeding for five days from *Brassica nigra* plants attacked by a sequence of herbivory with the guild or the species identity of first attacker constant but changing the order of the subsequent attackers of the sequence. First attacker: a) an aphid (A), b) a chewer (C), c) *Brevicoryne brassicae* (Bb) d) *Myzus persicae* (Mp), e) *Plutella xylostella* (Px), f) *Athalia rosae* (Ar). Boxplot height corresponds to the first and third quartiles (Q1 and Q3), and the middle line to the median. Asterisks show with significance differences * $P < 0,05$.



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 lose 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.

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CHAPTER 7
General Discussion

INTRODUCTION

In terrestrial ecosystems, plants are at the basis of many food chains. Plants are the food source, mating place, or a shelter to hide from predators for many organisms. Consequently, plants are central in food webs and mediate many direct and indirect interactions between organisms. Insects are one of the most important groups of organisms interacting with plants. It is estimated that there are up to five million species of insects on Earth, of which 50% are herbivores (Schoonhoven, van Loon, Dicke, 2005). Therefore, interactions with insect herbivores are very common, and represent a strong selection force on plant traits. Insects represent one of the most diverse groups of organisms, and this diversity is reflected in the array of strategies they have evolved to exploit plants. Given that in nature plants are under the attack by multiple herbivores, plants also evolved a repertoire of defence strategies to prevent their tissues from being consumed. Defending against multi-herbivore attack is not an easy task, as it implies being able to counteract a diversity of stressors that range from a microscopic bacterium to a big mammal.

In my PhD project, I explored how *Brassica nigra* plants deal with individual or simultaneous attack by its most common herbivores. In this chapter I will discuss: 1) The molecular regulation of how plants deal with multi-herbivore attack; 2) Challenges for plants of dealing with multi-herbivore attack; 3) Specificity of plant phenotypic changes induced by herbivory; 4) Plant strategies to optimise defence responses to multiple attackers; 5) Ecological consequences of induced plant responses to multiple attack; I end this general discussion with a perspective for future research.

MOLECULAR REGULATION OF HOW PLANTS DEAL WITH HERBIVORE ATTACK

Plants interact with a diverse community of attackers that may all require different defensive traits. Therefore, plants are under selection to recognize the specific attacker by its feeding guild (e.g. leaf chewer or phloem feeder), feeding pattern, feeding position, or elicitors in its saliva and mount a defence



response. Induced defence theory posits that plants tailor the induced-defence phenotype to the specific attacking herbivore (Erb *et al.*, 2012; Kliebenstein, 2014). However, how specific the plant phenotype is to the attacker remains to be investigated. My work has identified that some of the molecular responses of *B. nigra* to herbivore attack are **attacker specific**, although there are **guild-specific responses** as well (**chapter 3** and **chapter 4**). In chapter 3 I show that, in line with many studies that show that chewers induce JA (Erb *et al.*, 2012), *B. nigra* plants induce the expression of a JA-marker gene upon attack by the chewers *Athalia rosae*, *Pieris brassicae* and *Plutella xylostella*. In contrast, the expression of a JA-marker gene was not affected by the chewers *Autographa gamma*, *Mamestra brassicae* and *Pieris rapae*. This lack of JA induction by the latter chewers could be attributed to a limited leaf consumption by those insects, or to a site-dependent induction, as has been reported in other studies (Bonnet *et al.*, 2017; Chen *et al.*, 2020; Chrétien *et al.*, 2018). In **chapter 3** I show that, in line with literature (Appel *et al.*, 2014), chewers induce a JA-biosynthetic gene, and phloem feeders induce a SA responsive gene. However, this was only true when the chewers were specialists as generalist chewers did not induce JA compared to phloem feeders. The expression of an SA-responsive gene was lower in plants attacked by generalist leaf chewers than in plants induced by specialist and generalist phloem feeders, whereas it did not differ between specialist chewers and phloem feeders. Thus, I show that the molecular responses to single attack partly depend on the degree of specialisation of the attacker.

In **chapter 4**, I went one step further, and challenged plants with one, two or four species of chewing insects, phloem-feeding insects or a combination of both. Compared to undamaged plants or to plants that were attacked by phloem feeders, **all the chewers tested induced the JA-biosynthetic gene (*LOX2*) as well as the JA-responsive gene (*VSP2*)**. In contrast with **chapter 3**, where I found that feeding by the chewer *M. brassicae* did not induce JA, in **chapter 4**, *M. brassicae* induced the expression of two JA-related genes. This inconsistency between the two chapters could be explained by the differences in the set-up for the molecular analysis. While in **chapter 3** the insects were allowed to freely feed



from the plant and the youngest fully-expanded leaf was sampled for genetic analysis, in **chapter 4** the insects were constrained to one leaf, and that same leaf was sampled. As *M. brassicae* has a very spread feeding pattern (personal observation), the leaves sampled for chapter 3 likely had lower local induction than the ones sampled for chapter 4. This indicates how the methodology can influence molecular results and the importance of taking this into consideration when designing experiments. Even though in **chapter 4** I show that all chewers tested induced JA-regulated responses, I also show **specificity** in the response to species or their combination. I found that simultaneous attack by two chewers induced *LOX2* and *VSP2* more strongly than single attack by one chewer, than simultaneous attack by four chewers or than attack by a mix of chewers and phloem feeders (Fernández de Bobadilla *et al.*, 2021). Similarly, *Brassica juncea* plants simultaneously attacked by two chewers had a stronger defence induction (alkenyl glucosinolate production and trichome density), compared with plants under single attack (Mathur *et al.*, 2013).

The current paradigm is that plant responses to phloem-feeder attack are regulated via the SA-pathway (Erb *et al.*, 2012; Jaouannet *et al.*, 2014; Züst & Agrawal, 2016). Moreover, it has been proposed that the phloem-feeder suppression of JA could be due to negative crosstalk between the JA and SA pathways (Soler *et al.*, 2012; Züst & Agrawal, 2016). However, experimental evidence for SA/JA antagonism remains inconsistent (Broekgaarden *et al.*, 2011; Y. Li *et al.*, 2016). In line with the **JA/SA negative crosstalk paradigm** (Johnson *et al.*, 2020; Schweiger *et al.*, 2014; Zarate *et al.*, 2007) I show in **chapter 4** that feeding by one or by four species of phloem feeders suppressed the expression of a JA-responsive gene compared with undamaged plants, or with plants treated with chewers or with a mix of chewers and phloem feeders. Nonetheless, in contrast with the studies that claim that phloem feeders induce SA, in **chapter 3** and **chapter 4** I show that in *B. nigra*, the expression of the SA-responsive gene (*PR1*) was not induced by any of the phloem feeders, compared to expression levels of these genes in undamaged plants. Surprisingly, the only study that investigated *B. nigra* transcriptomic responses to phloem-feeder attack, shows that the responses



involve the JA and the ET pathway (Broekgaarden *et al.*, 2011). The expression of the SA marker gene (*PR1*) was induced by chewers and by a mix of chewers and phloem feeders (**chapter 4**). In contrast with my results, another study on *B. nigra* found that feeding by the chewer *P. rapae* did not affect expression of the SA-marker gene *PR1* (Broekgaarden *et al.*, 2011). However, other studies reported SA and JA induction upon leaf-chewer feeding (Arena *et al.*, 2018; Heidel & Baldwin, 2004; Kielkiewicz *et al.*, 2019; Y. Li *et al.*, 2016). This disparity in results might be attributed to different induction times. Interestingly, in **chapter 4** I show that the phloem-feeder suppression of JA-responses also occurs when phloem feeders and chewers simultaneously attack the plant. The expression of two JA-marker genes was lower on plants under simultaneous attack by phloem feeders and chewers, compared to plants only attacked by chewers, which might be interpreted as JA/SA negative crosstalk. The molecular regulation of plants simultaneously attacked by insects of different feeding guilds has rarely been investigated. The few studies available show that when phloem feeders are introduced to the plant together with chewers, many genes are suppressed compared to single-guild attack (Rodríguez-Saona *et al.*, 2010).

Thus, plant molecular regulation of responses to herbivore attack is not as simple as chewer induced-JA/phloem feeder induced-SA. Some studies have investigated plant transcriptomic responses of plants under dual attack (Bonnet *et al.*, 2017; Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016; Kroes *et al.*, 2017). However, the molecular regulation of plants attacked by more than two herbivores (i.e. multi-herbivore attack) has never been explored. Further research should investigate the molecular regulation of plant responses to multiple herbivores of the same or of different feeding guilds that arrive sequentially, as well as the role of herbivore instar, plant ontogeny, combination of stresses (both biotic and abiotic). In this respect, transcriptome analysis of plants under multi-herbivore attack will give a more complete view of the molecular regulation of plants against multiple attackers and give some hints on important regulators in defence against phloem feeders, chewers or a mix of both. Additionally, the molecular regulation of simultaneous attack by insects



of different feeding guilds may be different across plant ontogeny, plant species or depend on attacker identity.

CHALLENGES IN DEALING WITH MULTI-HERBIVORE ATTACK

Plant resistance to herbivory is modulated by the identity (Agrawal *et al.*, 2014; Van Zandt & Agrawal, 2004), guild (Bidart-Bouzat & Kliebenstein, 2011; Davidson-Lowe *et al.*, 2019), and the density of the attacker (Kroes *et al.*, 2016; Kroes *et al.*, 2015; Pineda *et al.*, 2017; Ramirez & Eubanks, 2016), among others factors. Only recently, studies including more than two attackers have unravelled that the order of attackers also influences a plant's ability to defend against attackers (Stam *et al.*, 2017, 2018, 2019; Viswanathan *et al.*, 2007). However, many aspects of multi-herbivore attack remain to be investigated. Among them, the role of herbivore richness on plant defence had never been investigated. In **chapter 4**, I show that the richness of attackers as well as their feeding guild determine a plant's ability to defend against herbivores. Increased richness in phloem feeders compromises plant resistance to a chewer. Plants simultaneously attacked by four species of phloem feeders become more susceptible to a chewer, whereas this is not the case for plants attacked by one or by two species of phloem feeders. The lack of induced susceptibility to a chewer by one or by two species of phloem feeders in *B. nigra* contrasts with previous studies on other plant species (Soler *et al.*, 2012) and shows that responses to herbivores are often plant species-specific (Hervé & Erb, 2019; B. Li *et al.*, 2018). In line with previous studies reporting chewer-induced resistance to a chewer, in **chapter 4** I show that leaf chewers induce resistance to subsequent attack by a chewer. Interestingly, chewer richness does not play a significant role in chewing herbivore induced responses. Attack by a mix of herbivores from different feeding guilds results in plant resistance similar to resistance levels of plants that were not previously exposed to herbivory. Very few studies investigated resistance of plants attacked by insects of two different feeding guilds and, in line with my results, these studies generally show an attenuation on plant defence traits on mixed induced plants, compared to



chewer induced plants (Ali & Agrawal, 2014; Delphia *et al.*, 2007; Rodriguez-Saona *et al.*, 2003, 2005; Rodriguez-Saona *et al.*, 2010). The absence of induced susceptibility or induced resistance in plants attacked by a mix of chewers and phloem feeders was true for most of the combinations of chewers and phloem feeders.

In addition to herbivore richness, I have unravelled that history of sequential herbivore attack differentially affects a plant's capability to defend itself against a subsequent herbivore (**chapter 6**). 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 influences plant resistance to subsequent herbivory.

In summary, in this thesis I have shown that the natural prevalence of herbivores, feeding guild, their identity, richness and the order of the attackers affect plant ability to defend against a late arriving herbivore. My thesis shows that to understand the evolution of induced defence strategies plant responses to multi-herbivore attack should be evaluated in a community context including sequential herbivores of different feeding guilds. The role of herbivore instar, plant ontogeny, plant species and combination of stressors (both biotic and abiotic) remains to be investigated.

SPECIFICITY OF PLANT PHENOTYPIC CHANGES INDUCED BY HERBIVORY

The molecular mechanisms to defend against attackers regulate the expression of plant phenotypic traits. Among the most important traits to defend against herbivores are physical barriers, secondary metabolites and defence proteins. Importantly, defence traits can be induced or repressed upon herbivory (Karban, 2011). Many studies claim that plants tailor their response to the specific attacker. There is extensive evidence for specificity in plant recognition of the attacker (Santamaria *et al.*, 2021), however, the proof



for specificity in plant response in terms of plant phenotype is lacking (Erb *et al.*, 2012). By studying the performance of a second attacker on plants induced with different herbivore treatments, in this thesis I studied some components of specificity of plant-induced responses to herbivory. In **chapter 3**, I show that even though the molecular plant responses to herbivores are specific to the feeding guild of the attacker, the induced resistance to a second attacker is not specific to the feeding guild nor to the identity of the first attacker. Strikingly, the induced resistance to one herbivore is better explained by the prevalence of the second herbivore in the field (Mertens *et al.*, 2021). By showing that feeding guild of the initial attacker is not explaining resistance to a second attacker I challenge the current paradigm on plant limitations to deal with sequential attack by insects of different feeding guilds (Moreira *et al.*, 2018; Thaler *et al.*, 2012). A general defence phenotype that makes the plant resistant to the more common attackers is likely more adaptive than a tailor-made response to every single attacker. In **chapter 4**, I challenged plants with 51 herbivore treatments of one, two, or four herbivore species of chewers, phloem feeders or a mix of both and studied resistance to caterpillars of *P. xylostella*, one of the most prevalent herbivores interacting with *B. nigra* plants (Mertens *et al.*, 2021). I found that there is some degree of **specificity**, even though all the chewer treatments induce a general JA-regulated response and make the plant more resistant to a second attacker, compared with untreated plants (Fernández de Bobadilla *et al.*, 2021). Plants simultaneously attacked by *A. rosae* and *Phaedon cochleariae* were more resistant to a second attacker than plants simultaneously attacked by the four chewers (*A. rosae* – *M. brassicae*–*P. brassicae*–*P. cochleariae*). Likewise, in a different study *Brassica juncea* plants simultaneously attacked by two chewers had a strong defence induction (alkenyl glucosinolate production and trichome density), compared with plants under single attack (Mathur *et al.*, 2013).

Many studies indicate that, although attacker recognition, and the molecular regulation of the response are specific, the final plant phenotype in response to very different organisms is similar. One striking example is the



response of *Arabidopsis* plants to insect eggs, being very similar to PAMP-triggered response to pathogens (Gouhier-Darimont *et al.*, 2013; Hilker & Fatouros, 2015). Probably, plants do not have a regulatory network to respond to every unique organism, but rather an integrated network that orchestrates the response to multiple attackers. Likely plants are equipped with a suite of defence traits, and under herbivory induce the most effective combination of traits to minimize the fitness loss. An important question to ask is why there is specificity in recognition if there is not specificity in response. For example, several studies show that plants can differentiate between mechanical damage, damage by chewing insects, and between insect-associated cues (frass, vibrations) (Acevedo *et al.*, 2015; Appel & Coccoft, 2014; Body *et al.*, 2019; Ray *et al.*, 2015) but the changes in phenotype to these cues are not so specific. One explanation would be that a general recognition system to differentiate between self and non-self is not enough to tell apart beneficial from detrimental organisms. However, a general response is probably effective against most of the attackers. Plants interact with phylogenetically very similar microorganisms that can range from beneficial to pathogenic. To avoid letting a pathogen in, mounting a hypersensitive response against a beneficial microorganism, plants need a very specific recognition system. Actually, when looking at plant interactions with beneficial microorganisms, initial recognition of MAMPs by the plant results in a mild induction of immune responses (Van Wees *et al.*, 2008) and to have a successful colonisation, the microorganisms need to manipulate the plant immune system (Zamioudis & Pieterse, 2012). Thus, plant responses to different attackers/organisms can be very specific and also be changed by the organism themselves, generating an extensive array of multiple scenarios for plant responses.

PLANT STRATEGIES TO OPTIMISE DEFENCE RESPONSE TO MULTIPLE ATTACKERS

The big diversity of herbivores and ways they exploit plants challenges plants to express an optimal defence phenotype. On the one hand, plants may be genetically limited, and lack the specific genetic pathway to produce



the appropriate response against a specific insect. In other cases, plants may have the defence pathway to target one insect, but the limitation comes in terms of antagonistic crosstalk between phytohormones. This negative communication may impair plant responses to one insect when the plant has already responded to a different insect. Importantly, insects are sensitive to different plant traits and often ecological trade-offs occur, where by gaining resistance to one herbivore plants are more vulnerable to others, or become less attractive to beneficial insects such as natural enemies of the herbivores and pollinators. To mention some of the challenges plants face, herbivore attack may vary in terms of the type of attacker (i.e. feeding guild or identity), the timing and order of arrival, the abundance, species richness, and plant parts attacked. The high diversity of stressors surrounding plants, makes it imperative that plants have evolved several strategies to survive. Plants need to use their resources efficiently, and accurately, and the strategy played will depend on the combination and type of stressors they are facing. In **chapter 2**, I propose several strategies that plants may have, to deal with a dynamic community of multiple attackers (Fig. 1). These defence strategies could be seen as risk management tools to deal with a multitude of stressors. In this thesis I identify some of the strategies listed in *B. nigra* plants. Additionally, I have found evidence from other studies that show the strategies in other plant species.

I show that *B. nigra* plants *canalise* their defence response to the first attacker as chewers induce resistance to a chewer and phloem feeders induce vulnerability to a chewer (**chapter 4**). The *canalisation* strategy has been reported in several plant species belonging to diverse families such as the Brassicaceae, Apocynaceae, or Solanaceae (Ali & Agrawal, 2014; Davidson-Lowe *et al.*, 2019; Soler *et al.*, 2012). However, under multiple attack by attackers arriving in sequences, *B. nigra* plants did not *canalise* their phenotype to the first attacker, as there is no induced susceptibility to a herbivore after sequences of four herbivores, compared with undamaged plants (**chapter 6**). In **chapter 4**, plants were attacked by the herbivores simultaneously, whereas in **chapter**



6 the herbivores were introduced in sequences of attack by four herbivores. During the growing season of *B. nigra*, plants receive sequential attack by many herbivores, and therefore the herbivore treatments applied in **chapter 6** resemble more closely the natural situation. The induced susceptibility after being simultaneously attacked by four species of phloem feeders could be an artefact, as this situation barely happens for *B. nigra* in natural ecosystems. A more common situation in nature would be *B. nigra* plants being attacked by a mix of chewers and phloem feeders, and in this situation, there was no herbivore-induced susceptibility (**chapter 4**).

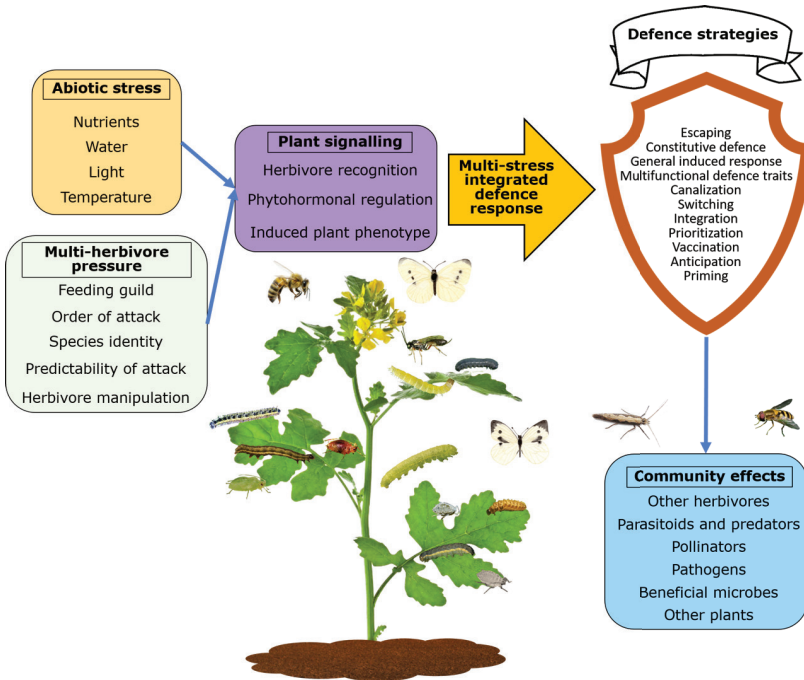


Figure 4. Plants experience multiple biotic and abiotic stresses during their lifespan. All these stresses are expected to be integrated and will determine which defence strategy is played by the plant.



Brassica nigra plants *integrate* responses to two different attackers when simultaneously attacked by chewers and phloem feeders, as plants showed an intermediate phenotype compared to plants attacked by either chewers or phloem feeders (**chapter 4**). Likewise, other plant species *integrate* responses to double attack by chewers and phloem feeders, and attenuate JA-related responses to chewers in the presence of phloem feeders (Ali & Agrawal, 2014; Kielkiewicz *et al.*, 2019). Additionally, **chapter 6** suggests that *B. nigra* plants do not fully *switch* their resistance phenotype to a new attacker. 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 phloem feeder– chewer – phloem feeder– chewer than on plants attacked by the sequence phloem feeder– phloem feeder– 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. In contrast, *Arabidopsis thaliana* plants under sequential stress are able to *switch* their defence machinery towards the second stress (Coolen *et al.*, 2016). Transcriptomic analysis of *A. thaliana* during sequential biotic and abiotic stresses showed that even though individual stresses triggered very different transcriptomic profiles, when two stresses were applied in sequence, plants displayed transcriptome profiles that were very similar to the second stress, irrespective of the nature of the first stress (Coolen *et al.*, 2016). Evidence shows that plants can remobilize and reallocate resources from secondary to primary metabolism and vice versa (Erb & Kliebenstein, 2020). Probably this is also the case in the context of *switching* the response to different attackers where plants could produce chemicals in response to the current attacker, and when a different attacker comes, and it needs another response, reabsorb the first compound and use it to produce new defence compounds (Bekaert *et al.*, 2012). Biosynthetic enzymes and intermediates are shared between pathways (Tholl *et al.*, 2005), reducing the costs of *switching* responses between attackers. Also, multi-functional enzymes with broad substrate specificity may reduce costs of switching because fewer intermediates are required (Tholl *et al.*, 2005).



Among the plant-associated herbivore community members, some attackers are more damaging than others (Poelman & Kessler, 2016) and plants may use the *prioritization* strategy, and wait for their attack to induce a defence response. This strategy can also be used to *prioritize* defence of tissues which loss imply a high fitness cost (e.g. flowers) (Chrétien *et al.*, 2018; Keith & Mitchell-Olds, 2017). Strikingly, I show that *B. nigra* plants *anticipate* and prepare for the attack of the more damaging herbivores (**chapter 3**) as plants are more resistant to sequential attack by herbivores that are more common in the field (Mertens *et al.*, 2021). Plants responding to insect cues (damage or egg deposition) and inducing resistance against future attack are using the *anticipation* strategy as well (Dalin & Björkman, 2003; Orrock *et al.*, 2018; Pashalidou *et al.*, 2015). Other studies have shown plants to be equipped with the *vaccination* strategy inducing a response to a herbivore species that can be tolerated with the benefit of reduced attack by a more ravaging herbivore (Kessler & Baldwin, 2004). Some authors even claim that some environmental cues can *prime* plants and prepare them for future attack (Conrath *et al.*, 2006; Martínez-Medina *et al.*, 2016).

The array of plant strategies against insect herbivores that I identified in **chapter 2**, are likely involved in defence against other herbivores as well. As in nature plants encounter simultaneously or sequentially very diverse biotic and abiotic stressors, the optimal phenotype will likely include integration of resistance to all of these stressors. Presumably, plants are equipped with a defence network machinery that integrates responses to individual stresses. In the past years a few studies have started exploring plant transcriptomic responses to stressors of different nature. Undoubtedly, the plant regulation of defence against a combination of stressors is very different from that against a single stress (Coolen *et al.*, 2016; Davila Olivas *et al.*, 2016; Davila Olivas, Kruijer, *et al.*, 2017). However, we are far from understanding the regulatory network underlying responses to a combination of attackers. More importantly, there is no evidence on whether the induced plant phenotypic changes are -as many studies claim- “tailor-made” to the stressor, or rather a general phenotype that targets all of them (Jones & Dangel, 2006; Voelckel & Baldwin, 2004; Vogel *et al.*, 2007).



Presumably, the expressed plant phenotype does not target individual stressors. It has already been shown that plant traits against slugs often make plants more resistant against insects (Calf *et al.*, 2018; Hanley & Sykes, 2009; Hanley *et al.*, 2018; Loy *et al.*, 2017; Roda *et al.*, 2019). There is also extensive evidence showing that pathogens and insects interact via plant-mediated changes (Badri *et al.*, 2013; Desurmont *et al.*, 2016; Eberl *et al.*, 2018, 2020; Franco *et al.*, 2017; Guo *et al.*, 2014; Hauser *et al.*, 2013). Therefore, a defence strategy making plants resistant against both insects and pathogens is likely adaptive. It has even been shown that plants use identical inhibitors to restrict insect feeding and pathogen infection (Kirsch *et al.*, 2020). In order to truly understand plant-trait selection, future studies should embrace the complexity of plant interactions with all stressors and friends. Exciting discoveries on plant strategies to their biotic and abiotic environment are expected to result from this.

Given the evidence of plants having several strategies to counteract herbivory, the pertinent question to ask is whether individual plants are equipped with a full set of strategies or only have a few. Additionally, we should question if plants that have evolved under different herbivore communities have similar strategies (Davila Olivas *et al.*, 2017). If plants lack any of the strategies the question is why selection has eliminated some, and on which basis. Possibly, plants exhibit different strategies depending on the situation (i.e. herbivore pressure and abiotic stress), and can even exhibit different strategies simultaneously in different plant parts. For example, plants may exhibit local *canalisation* (Chen *et al.*, 2020) in one leaf attacked by a very damaging herbivore and *integration* in another leaf attacked by two different herbivores.

ECOLOGICAL CONSEQUENCES OF PLANT INDUCED RESPONSES TO MULTIPLE ATTACK

In natural ecosystems, plants are central in a network of species that interact directly or indirectly via the plant (Ohgushi, 2005). Herbivores induce dramatic changes in a plant phenotype and alter the way plants are perceived by other organisms thereby altering community dynamics. Importantly,



herbivore-induced changes in plant phenotype strongly affect the foraging of later arriving herbivores, parasitoids and pollinators (Chrétien *et al.*, 2021; Rusman *et al.*, 2018; Soper Gorden & Adler, 2018). In **chapter 5**, I show that plants treated with herbivores were more attractive to a later arriving herbivore in the field. Likewise, in a field study *P. xylostella* was more abundant on *Brassica oleracea* plants attacked by *P. rapae*, compared with undamaged plants (Poelman *et al.*, 2008). Strikingly, in my thesis I show that the positive effect of herbivory on colonisation by a later herbivore is maintained regardless of the guild and the richness of species of the initial herbivores (**chapter 5**). The consistently higher abundance of *P. xylostella* on herbivore-treated plants could be attributed to a herbivore-induced change in important plant traits for insect foraging. One possible explanation for *P. xylostella* preferring herbivore-induced plants is that the herbivore-damaged plants might be more detectable because of a higher HIPV emission (El-Sayed *et al.*, 2016). Alternatively, plants that received herbivory might represent an enemy-free space for *P. xylostella* (Dicke *et al.*, 2009; Stamp, 2001). In my thesis I have not recorded differences in *P. xylostella* colonisation between the investigated herbivore combinations in the field (**chapter 5**). This **lack of specificity**, partly contrasts with my findings of **chapter 4**, where *P. xylostella* performs better on plants attacked by the highest species richness of phloem feeders, whereas the performance was reduced on plants attacked by chewers, irrespectively of the richness of attackers (Fernández de Bobadilla *et al.*, 2021). Further research should disentangle which plant traits mediate the differences in *P. xylostella* performance found in **chapter 4**, that do not correspond with the difference in abundance found in **chapter 5**.

Importantly, herbivore-induced changes in plant traits can also affect higher trophic levels. However, in contrast to the consistently stronger abundance of *P. xylostella* in herbivore-treated plants, compared to untreated plants the parasitism of *P. xylostella* was not higher in all the herbivore treated plants, and it was higher in some of the herbivore-treated plants than in others (**chapter 5**). Thus, I found specificity in the herbivore-induced changes in



important cues for parasitoid foraging. Other studies found that parasitoids are able to discriminate between plants that have been attacked or not, between plants under single or dual attack, and even on the type and density of attackers (Blubaugh *et al.*, 2018; Bukovinszky *et al.*, 2012; De Boer *et al.*, 2008; de Rijk *et al.*, 2016; De Rijk *et al.*, 2013; Ponzio *et al.*, 2016; Rodriguez-Saona *et al.*, 2003; Rodriguez-Saona & Thaler, 2005). Nonetheless, the importance of species richness and traits for parasitoid attraction under field conditions was never explored. Interestingly, the **specificity in parasitism rate** that I report in **chapter 5**, was not explained by the guild of the attackers. Compared with untreated plants, I found a higher degree of parasitism not only on plants attacked by some of the chewers, but also on plants attacked by some of the phloem feeders. The highest parasitism rate was found on plants induced by a mix of two chewers and two phloem feeders. One potential explanation for the differential attraction of parasitoids to mixed-induced plants compared to single-guild induced plants is the effect that the mixed induction may have on the volatile blend of the plant. For example, cotton plants under dual attack by caterpillars of *Spodoptera exigua* and by the phloem-feeding insect *Bemisia tabaci* emitted lower amounts of volatiles compared with plants only attacked by caterpillars (Rodriguez-Saona *et al.*, 2003). Presumably depending on the herbivore species in the mix, and on the parasitoids tested as responder, the attraction is enhanced or reduced (Dicke *et al.*, 2009). The higher attraction towards mixed-induced plants from our study could be due to a higher detectability of the volatiles from mixed-induced plants (Aartsma *et al.*, 2019). Additionally, the parasitoids may associate the mixed-induced volatiles with a higher quality of the host. In **chapter 4**, I showed that compared to the performance on undamaged plants, *P. xylostella* grew less on plants induced by chewers, while on mixed-induced plants the performance was not reduced (Fernández de Bobadilla *et al.*, 2021).

On top of the herbivore-induced effects on later arriving herbivores and parasitoids, pollinator visitation to *B. nigra* plants changes depending on the feeding guild, and the species richness of the herbivores attacking the plant



(chapter 5). Previous research on effects of single herbivore-induced changes on pollinator visitation show that the pollinator community of *B. nigra* differed depending on whether plants were uninfested or infested with a chewing herbivore, but it did not change when they were attacked by phloem feeders (Rusman *et al.*, 2018). In contrast, I found that the pollinator community is also affected by phloem-feeder infestation, compared to the community visiting flowers on untreated plants (chapter 5). Interestingly, not only the richness of herbivores affected the pollinator visitation, but also species identity. While in general herbivory affects pollinators, some of the changes depend on the herbivore species. This herbivore-specificity contrasts with other studies that could not detect differences in the pollinator visitation between plant treatments with individual herbivore species (Rusman *et al.*, 2018). The changes in pollinator visitation found in our study may be mediated by herbivore-induced changes in floral traits, e.g., flower phenology, flower number and morphology, presence of deterrents in pollen and changes in flower volatiles (Kessler & Chautá, 2020; Moreira *et al.*, 2019).

In my thesis I have shown that herbivory affects plant interactions with other herbivores, with higher trophic levels and with pollinators (chapter 5). Importantly, I show that this change in interactions has consequences for plant fitness. Other studies reported herbivore-induced effects on plant fitness in the field (Cozzolino *et al.*, 2015; Devegili & Chalcoff, 2020; Lankau & Strauss, 2008; Soper Gorden & Adler, 2018). However, very rarely has it been studied how plants maximise fitness when defending against attack by diverse assemblages of herbivores while maintaining the attraction of parasitoids/predators and pollinators. I show that the composition of the introduced herbivore community affected plant fitness directly via reducing plant biomass that predicted resource investment into reproductive organs (chapter 5). Interestingly, the herbivore-induced changes in plant biomass were stronger than fitness consequences of herbivore-induced plant-mediated interactions with other community members (chapter 5). Fitness effects of indirect interactions often included a network of multiple organisms and could be mediated by plant traits. I show that all



of these interactions involved effects of herbivores on pollinators that closely connected with the total number of seeds produced by a plant (**chapter 5**). For a species-rich community of phloem feeders as well as for the leaf-chewer-rich community seed set was reduced via a chain of interactions that reduced pollinator visitation.

My work has unravelled that herbivore richness, functional traits and species identity alters direct and indirect interactions with other insects and has fitness effects on plants. This allows me to predict that selection on plant traits incorporates the effects of these interactions on plant fitness. However, due to the complexity and high connectivity of the interaction network it is very difficult to make predictions on directions of effects for plant-trait selection (Strauss, 2014). In many study systems diffuse evolution of plant traits has been reported, where selection on traits is not only explained by one-to-one species interactions, but rather is the effect of the network of interactions (Poelman & Kessler, 2016; Strauss, 2014).

CONCLUDING REMARKS AND FUTURE PERSPECTIVE

In conclusion, my thesis provides answers to important questions on plant responses to multi-herbivore attack. I show that plants tailor induced defence strategies to deal with common patterns of sequential herbivore attack and anticipate arrival of the most prevalent herbivores. Additionally, I show that *B. nigra* plants channel their defence responses stronger towards a feeding-guild specific response when under multi-species attack by herbivores of the same feeding guild, but integrate responses when simultaneously confronted with a mix of herbivores from different feeding guilds. Importantly, under field conditions, I show that herbivore richness, functional trait and herbivore identity affect plant fitness and plant interactions with a herbivore, parasitoids and pollinators. Finally, I show that history of sequential attack is an important factor determining plant resistance to herbivores.



Integrating field- studies with mechanistic physiological and molecular biology

In the last years we have been collecting knowledge on plant defences against stressors. All these studies, including my thesis, have helped us understanding some of the plant systems involved in stress detection, and the strategies to regulate an appropriated response. Nonetheless, how plants integrate information derived from multiple (i.e. more than two) stressors remains an open question. Moreover, how specific the induced plant phenotype is to the attackers remains to be further investigated. Future research should focus on unveiling the mechanisms that regulate plant responses to a combination of attackers. For such experiments a tight collaboration between ecologists and molecular biologists is needed. On the one hand, ecologists can provide valuable information on the type of interactions that happen in nature. On the other hand, molecular biologists can use this information to design ecologically meaningful experiments, where plants are challenged by stresses that they would experience in their natural environment. Investigating plant defence against their natural stressors is the only way that allows us inferring conclusions on the agents involved in plant-trait selection. These multi-disciplinary projects are a big -yet exciting- challenge that plant scientists should embrace.

Climate change and insect decline: effect on plant trait selection

In this thesis I have shown the importance of studying plant responses to multiple herbivores. First, I show that even plant physiological responses to multiple attack have some similarities with responses to single attack, important changes arise if more players are introduced. Second, I show that this is also the case for plant-mediated interactions between herbivores. A plant's ability to defend against herbivores is influenced by the history of multi-herbivore attack. Third, I have shown how multi-herbivore attack affects plant fitness and plant interactions with herbivores, parasitoids and pollinators. Given the current situation of global warming and insect extinction (Hallmann *et al.*, 2020; Wagner *et al.*, 2021), where many interactions between plants and insects



are being altered, I predict that dramatic changes in plant-insect interactions are already happening. This change in interactions poses great challenges to ecologists and agricultural scientists. Undoubtedly, scientists will embrace the challenge and guide society to prevent ecological catastrophes (Harvey *et al.*, 2020). In this context, research on plant interactions against multiple attackers can provide very relevant information. On one hand, it can help us predicting cascading effects on the plant-associated community, information that can be used for many purposes, including insect conservation strategies. On the other hand, as in agriculture crops are under the attack of multiple pests, designing of crop-protection practices can learn from fundamental studies on plant defence strategies to multi-herbivore attack.

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Summary

In terrestrial ecosystems, plants are the food source, mating place, or a shelter to hide from predators for many organisms. Consequently, plants are central in many ecosystems and mediate many direct and indirect interactions between organisms. Insects represent one of the most important groups of organisms interacting with plants. It is estimated that there are up to five million species of insects on Earth, of which 50% are herbivores. Therefore, interactions with insect herbivores are not only very common, but also represent a strong selection force on plant traits. In nature, plants are under the attack by multiple herbivores that arrive simultaneously or sequentially. There is extensive knowledge on plant defence against single or dual attack. However, how plants defend themselves against the attack of multiple insects is poorly understood. Therefore, the **aim of my PhD project**, was to explore how *Brassica nigra* plants deal with multi-herbivore attack by its most common herbivores.

Insects represent one of the most diverse groups of organisms, and this diversity is reflected in the array of strategies with which they exploit plants. Plants recognize the attackers by their feeding guild and by elicitors in the saliva and induce a defence response tailor-made to the specific attacker. Although most plants are under attack by a large diversity of herbivores, surprisingly little is known about the physiological capabilities of plants to deal with multi-herbivore attack. Studies on dual herbivore attack identified that defence to one attacker may cause energetic and physiological constraints to deal with a second attacker. How these constraints shape plant plasticity in defence to multi-herbivore attack and what defence strategies plants may deploy to their full community of attackers is a major knowledge gap in plant science. In **chapter 2**, I provide a framework for plant defence to multi-herbivore attack by defining the repertoire of plastic defence strategies that may allow plants to optimize defence against a multitude of stressors.

Plants have evolved plastic defence strategies to deal with uncertainty of when, by which species and in which order attack by herbivores will take place. However, due to antagonistic cross-talk between phytohormones, induced plant



responses to current herbivore attack may come with a cost of compromising resistance to other, later arriving herbivores. Studies on dual attack, show that the feeding guild of the initial herbivore is considered to be the primary factor determining whether resistance to subsequent attack is compromised. In line with the current paradigm of phytohormonal regulation of plant defence to insect herbivory, in **chapter 3** I show that plant responses to chewers and phloem feeders are regulated via the SA and JA pathways, respectively. However, also in **chapter 3**, by investigating 90 pair-wise insect-herbivore interactions among ten different herbivore species, I show that resistance of the annual plant *B. nigra* to a later arriving herbivore species is not explained by feeding guild of the initial attacker. Instead, the prevalence of herbivore species that arrive on induced plants based on three years of season-long insect community assessments in the field explained cross-resistance. Plants maintained resistance to prevalent herbivores in common patterns of herbivore arrival and compromises in resistance especially occurred for rare patterns of herbivore attack. Therefore, plants tailor induced defence strategies to deal with common patterns of sequential herbivore attack and anticipate arrival of the most prevalent herbivores.

In **chapter 4**, I went one step further towards more natural conditions, and challenged plants with simultaneous attack by multiple herbivores. I subjected *B. nigra* plants to 51 treatments representing attack by an increasing species richness (1, 2 or 4 species) of either phloem feeders, leaf chewers, or a mix of both feeding guilds when keeping total density of attackers constant and studied how this affects plant resistance to subsequent attack by caterpillars of the diamondback moth (*Plutella xylostella*). I show that increased richness in phloem-feeding attackers compromised resistance to *P. xylostella*. In contrast, leaf chewers induced a stronger resistance to subsequent attack by caterpillars of *P. xylostella* while species richness did not play a significant role for chewing herbivore induced responses. Attack by a mix of herbivores from different feeding guilds resulted in plant resistance similar to resistance levels of plants that were not previously exposed to herbivory. Plant responses to all the chewers



and to all the combination of chewers were regulated via the JA-pathway. In line with the JA/SA negative crosstalk paradigm I show that feeding by one or by four species of phloem feeders suppressed the expression of a JA-responsive gene compared with undamaged plants, or with plants treated with chewers or with a mix of chewers and phloem feeders. The expression of the SA-responsive gene (*PR1*) was not induced by any of the phloem feeders, compared to expression levels of these genes in undamaged plants.

In **chapter 5**, I studied how insect herbivore species richness and trait composition of these species affect plant interactions with other insect community members (i.e. a herbivore, its parasitoids and pollinators) and whether these interactions affect plant fitness. A main challenge in ecology is to assess how loss of species and consequently species interactions affect ecosystem stability. This knowledge is especially relevant within the context of global insect decline that planet Earth faces. I show that herbivore richness, its composition of functional traits and specific herbivore species affected the abundance of *P. xylostella*, the parasitism rate of *P. xylostella* and pollinator visitation to *B. nigra* plants. The composition of the introduced herbivore community affected plant fitness directly via reducing plant biomass that predicted resource investment into reproductive organs and these effects were stronger than fitness consequences of herbivore-induced plant-mediated interactions with other community members. Fitness effects of indirect interactions often included a network of multiple organisms and could be mediated by plant traits. All of these interactions involved effects of herbivores on pollinator visitation that closely correlated with the total number of seeds produced by a plant. For a species-rich community of phloem feeders as well as for the leaf-chewer rich community seed set was reduced via a chain of interactions that reduced pollinator visitation.

In **chapter 4** and in **chapter 5** I thus show that the richness of herbivores simultaneously attacking a plant influences a plant's ability to defend itself against a subsequent herbivore and to maintain interactions with mutualistic



members of the community. However, the question of how plants defend against the attack of multiple herbivores that arrive sequentially, remained unanswered. Therefore, in **chapter 6** I investigated whether *B. nigra* plants are able to defend themselves against caterpillars of the late arriving herbivore *P. xylostella*, when plants had been previously exposed to sequential attack by four other herbivores. I 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. I found that history of sequential herbivore attack differentially affected the capability of *B. 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, my thesis provides answers to important questions on plant responses to multi-herbivore attack. I show that plants tailor induced defence strategies to deal with common patterns of sequential herbivore attack and anticipate arrival of the most prevalent herbivores. Additionally, I show that *B. nigra* plants channel their defence responses stronger towards a feeding-guild specific response when under multi-species attack by herbivores of the same feeding guild, but integrate responses when simultaneously confronted with a mix of herbivores from different feeding guilds. Importantly, under field conditions, I show that herbivore richness, functional trait and herbivore identity affect plant fitness and plant interactions with a herbivore, parasitoids and pollinators. Finally, I show that history of sequential attack is an important factor determining plant resistance to herbivores.





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About the author

Maite Fernández de Bobadilla was born on July 21st, 1989 in Madrid, Spain. She grew up in Dénia, a very nice town at Alicante's coast. She studied Biology at València. The last year of her bachelor she joined an Erasmus program and moved to Prague. At that moment, she did not know that she would have to wait 8 years to come back to Spain. She lived one year in Augsburg, then moved to Wageningen to join the MSc program Plant Sciences with a focus on Entomology. For her first MSc thesis, supervised by Ana Pineda, she studied the role of drought on the interactions between plants, beneficial bacteria and an insect herbivore. She enjoyed this project so much that she decided doing a second MSc thesis, instead of an internship. She went to the Max Planck Institute in Jena, Germany. There, supervised by Dr Sybille Unsicker and Dr Franziska Eberl, she studied the interactions between poplar trees, a rust fungus and herbivore insects. This thesis was awarded with the East-West Seed Graduation prize for Plant Sciences. Her MSc program was awarded with the qualification of cum laude. In January 2017, she came back to Wageningen to join the team of Dr Erik Poelman as a PhD candidate. In her PhD project she studied plant adaptations to the attack of multiple insects. Maite did very diverse experiments, including greenhouse and molecular studies, behavioural assays and field work. The last year of her PhD Maite joined a training program to become a Biology teacher in Spanish secondary schools. At the beginning of March 2020, Maite came to València to do the practical and teach at a school. She was trapped in Spain due to the Covid-19 pandemic. Maite finished her training program and her PhD working from home, in València. For the future Maite aims at becoming a Biology teacher in Spain, with a strong emphasis on environmental awareness.





List of publications

PUBLISHED PAPERS

- 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>
- Eberl, F., **Fernandez de Bobadilla, M.**, Reichelt, M., Hammerbacher, A., Gershenzon, J., & Unsicker, S. B. (2020). Herbivory meets fungivory: insect herbivores feed on plant pathogenic fungi for their own benefit. *Ecology Letters*, 23(7), 1073–1084. <https://doi.org/10.1111/ele.13506>
- Aartsma, Y., Cusumano, A., **Fernández de Bobadilla, M.**, Rusman, Q., Vosteen, I., & Poelman, E. H. (2019). Understanding insect foraging in complex habitats by comparing trophic levels: insights from specialist host-parasitoid-hyperparasitoid systems. *Current Opinion in Insect Science*, 32, 54–60. <https://doi.org/10.1016/j.cois.2018.11.001>
- Fernández de Bobadilla, M.**, Friman, J., Pangesti, N., Dicke, M., van Loon, J. J. A., & Pineda, A. (2017). Does drought stress modify the effects of plant-growth promoting rhizobacteria on an aboveground chewing herbivore? *Insect Science*, 24(6), 1034–1044. <https://doi.org/10.1111/1744-7917.12477>

SUBMITTED PAPERS

- Mertens, D., **Fernández de Bobadilla, M.**, Rusman, Q., Bloem, J., Douma, J. C., & Poelman, E. H. Plant defence to sequential attack is adapted to prevalent herbivores. *Nature Plants*.
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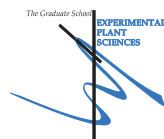
- Fernández de Bobadilla, M.**, Rusman, Q., Costaz, T., Alban, C., Arkoumanea, K., Mertens, D., & Poelman, E. H. Herbivore species richness influences plant interactions with an herbivorous insect, its natural enemies, and plant reproduction. *Ecology Letters*.





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Group: **Laboratory of Entomology**
University: **Wageningen University & Research**

1) Start-Up Phase	<i>date</i>	<i>CP</i>
▶ First presentation of your project Plant physiological plasticity to multitherbivore attack	05 Feb 2017	1,5
▶ Writing or rewriting a project proposal Plant adaptations to unpredictable attack by dynamic insect communities	Jan 2017	0,0
▶ Writing a review or book chapter		
▶ MSc courses		
<i>Subtotal Start-Up Phase</i>		1,5
2) Scientific Exposure	<i>date</i>	<i>CP</i>
▶ EPS PhD student days		
Annual EPS PhD Student Days Get2Gether, Soest, NL	09-10 Feb 2017	0,6
Annual EPS PhD Student Days Get2Gether, Soest, NL	15-16 Feb 2018	0,6
▶ EPS theme symposia		
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Wageningen, NL	23 Jan 2017	0,3
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Amsterdam, NL	24 Jan 2018	0,3
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', together with Willie Commelin Scholten Day, Wageningen, NL	01 Feb 2019	0,3
▶ Luneren Days and other national platforms		
Netherlands Annual Ecology Meeting (NAEM), Lunteren, NL	14-15 Feb 2017	0,6
29e Entomologendag 2017, Ede, NL	15 Dec 2017	0,3
31e Entomologendag 2019, Ede, NL	13 Dec 2019	0,3
▶ Seminars (series), workshops and symposia		
4th Annual Wageningen PhD symposium - Science: From local to global, Wag	03 May 2017	0,3
Insect-Plant-Interaction workshop, Wageningen, NL	07 Nov 2017	0,3
Insect-Plant-Interaction workshop, Leiden, NL	09 Oct 2018	0,3
Towards Ecological Intensification, the Importance of Biodiversity in Agricultural Landscapes	28 Aug 2019	0,3
Yearly Entomology Research Exchange Meeting (YELREM), Bennekom, NL	01 Jun 2017	0,3
Yearly Entomology Research Exchange Meeting (YELREM), Bennekom, NL	24 May 2018	0,3
Yearly Entomology Research Exchange Meeting (YELREM), Bennekom, NL	04 Jun 2019	0,3
WEES seminar: Prof. dr. Bregje Wertheim Evolving Immunity "Genomic basis of the evolution and variation in parasitoid resistance"	19 Jan 2017	0,1
WEES seminar: Dr. Mart Krupovic "Natural history of viral capsids"	23 Feb 2017	0,1
WEES seminar: Prof. Michael Singer "Host shift in butterflies"	04 Dec 2017	0,1
WEES seminar: Prof. Anne-Nathalie Volkoff "Endogenous viruses used by parasitic wasps to deliver virulence molecules to their hosts"	14 Dec 2017	0,1
WEES seminar: Alejandro Tena "Honeydew as food source for insects: a sweet that can become toxic"	30 Jan 2018	0,1
WEES seminar: Fred de Boer "Spatial aspects in disease ecology"	26 Feb 2018	0,1
▶ Seminar plus		
▶ International symposia and congresses		
16th Symposium on Insect-Plant Interactions, Tours, FR	02-06 Jul 2017	1,3
Spanish Applied Entomology Congress (SEEA), Madrid, ES	04-08 Nov 2019	1,2
8th IOBC Working Group Meeting "Landscape management for functional biod	27-29 Mar 2019	0,9
▶ Presentations		
Netherlands Ecological Annual Meeting 2017 (poster presentation)	15 Feb 2017	1,0
16th Symposium on Insect-Plant Interactions (poster presentation)	04 Jul 2017	0,0
Speaker at IVIA (Valencian Institute of Agrarian Research)	13 Nov 2018	1,0
Speaker at SEEA (Spanish Applied Entomology Congress)	04 Nov 2019	1,0
Speaker at 31e Entomologendag	13 Dec 2019	1,0
▶ IAB interview		
▶ Excursions		
EPS PhD Council Company Visit to Koppert	26 Oct 2018	0,2
<i>Subtotal Scientific Exposure</i>		13,6



3) In-Depth Studies	<i>date</i>	<i>cp</i>
▶ Advanced scientific courses & workshops		
Chemical Ecology, Jena, DE	27 Jun - 08 Jul 2016	3,0
Generalized Linear Models, Wageningen, NL	14-15 Jun 2018	0,6
Bugs at your service, Wageningen, NL	31 Mar - 5 Apr 2019	1,5
▶ Journal club		
Entomology, Wageningen, NL	2017-2021	3,0
▶ Individual research training		
<i>Subtotal In-Depth Studies</i>		8,1

4) Personal Development	<i>date</i>	<i>cp</i>
▶ General skill training courses		
PhD Competence Assessment, Wageningen, NL	Apr 2017	0,3
Scientific Artwork with Photoshop and Illustrator, Wageningen, NL	28-29 Apr 2017	0,6
EPS PhD Introduction Course, Wageningen, NL	26 Sep 2017	0,3
Scientific Writing, Wageningen, NL	02 Sep - 24 Oct 2019	1,8
Máster Universitario en Formación del profesorado de Educación Secundaria Obligatoria y Bachillerato, Formación profesional y Enseñanzas de idiomas, especialidad Biología y Geología (UNIR) (<i>Master for becoming a Teacher from Compulsory Secondary Education and Bachiller, Vocational Training and Language Teaching</i>)	Sep 2019 - Jul 2020	60,0
▶ Organisation of meetings, PhD courses or outreach activities		
▶ Membership of EPS PhD Council		
<i>Subtotal Personal Development</i>		63,0

TOTAL NUMBER OF CREDIT POINTS*	86,2
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS with a minimum total of 30 ECTS credits.	
* A credit represents a normative study load of 28 hours of study.	



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