Plant-mediated insect interactions on a perennial plant: consequences for community dynamics

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Voor Vaai en Oma

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Chapter 1

General introduction

Introduction

Plants are surrounded by many organisms with which they interact, ranging from neighbouring plants, soil microbes, large vertebrate grazers and tiny herbivorous, carnivorous or pollinating insects. As such, plants are at the basis of many terrestrial food webs (Price 2002; Schmitz *et al.* 2004; Schoonhoven *et al.* 2005), and by mediating interactions between species, plants are a central node in ecological interaction webs (Ohgushi 2005; Kaplan & Denno 2007; Utsumi *et al.* 2010). Insects form the most diverse above-ground players in the interaction web associated with plants (Schoonhoven *et al.* 2005; Stam *et al.* 2014). These interaction webs are highly dynamic over time, because both the plant phenotype and the insect community composition vary by reciprocal responses to each other (Karban & Baldwin 1997; Schoonhoven *et al.* 2005; Ohgushi 2016). A central question in ecology is therefore: How are these insect-plant communities structured and what determines their dynamics?

Variation in the phenotype of an individual plant occurs through responses to both the abiotic and biotic environment (Karban & Baldwin 1997; Schoonhoven *et al.* 2005; Karban 2011; Davila Olivas *et al.* 2016). When for example an herbivorous insect feeds from a plant, the plant can recognise insect damage and respond to this by changing its phenotype (Kessler & Baldwin 2004; Musser *et al.* 2012; Agrawal *et al.* 2014). These phenotypic responses can lead to altered tolerance or resistance of plants to herbivores (Hopkins *et al.* 2009; Karban 2011; Moreira *et al.* 2015) and this plant phenotypic plasticity creates a diverse landscape for insects associated with a plant (Karban & Adler 1996; Kessler 2015). In addition to insect phenology and plant genotype, plant phenotypic plasticity in response to insect herbivores is recognized as a major driver of the composition of the plant-associated insect community (Karban & Adler 1996; Kessler 2015; Ohgushi 2016).

The insect community that feeds on a plant can consist of several species that may have different feeding modes. Plants can respond specifically to induction by insects from different feeding guilds or even species (Bidart-Bouzat & Kliebenstein 2011; Soler *et al.* 2012a; Ali & Agrawal 2014). Phloem-feeders such as aphids induce a different biochemical signalling pathway in the plant than most leaf-chewers such as caterpillars (de Vos *et al.* 2005; Koo *et al.* 2013). These biochemical signalling pathways can interfere with each other through cross-talk of molecules from one pathway to another (Koornneef & Pieterse 2008; Pieterse *et al.* 2009). As a result, the plant phenotype can be variable upon induction by different herbivores. This variable plant phenotype affects a subsequent insect's choice to colonize a plant, as well as its survival and reproduction, which together determine the abundance of an insect species on a plant (Karban & Baldwin 1997; Schoonhoven *et al.* 2005; Kessler & Halitschke 2007).

Insect species that occur together on a plant may thus interact with each other, which occurs predominantly indirectly, mediated by the plant phenotype that is

induced by previous insects (Kaplan & Denno 2007). In this way plants can mediate interactions between species that are separated in space and/or time (Karban & Baldwin 1997; Ohgushi 2005; Utsumi *et al.* 2010). A subsequent insect on the plant, whether herbivore, carnivore or for example pollinator, may change the plant phenotype in its turn, which may again influence insect abundance and the dynamics of the whole community in a cascading manner (van Zandt & Agrawal 2004a; Ohgushi 2005; Viswanathan *et al.* 2005; Utsumi *et al.* 2010). Such continuous, dynamic interactions between plant and insects can cause evolutionary feedback loops (Utsumi 2011; Ohgushi 2016). The plant-mediated indirect interactions in an insect community may feed back to plant growth, its fitness in terms of the amount of biomass and seeds, and eventually even evolutionary changes in plant traits (Utsumi 2011; Turley & Johnson 2015; Utsumi 2015; Ohgushi 2016).

Variation in plant responses to multiple herbivores

Insects do not occur on a plant simply one after another as separate events, but multiple species may arrive simultaneously, or in quick successions in different orders of arrival. Multiple herbivore species on a plant may interfere with the cross-talk pattern between plant biochemical pathways in a different way than do single species (Zhang et al. 2009; Rodriguez-Saona et al. 2010). As a consequence, the plant phenotype after multiple herbivory can be different than the sum of responses to each herbivore alone (Soler et al. 2012a; Mathur et al. 2013; Zhang et al. 2013). Multiple herbivores on a plant thus have the potential to change insect-plant interactions, but we know only little of the consequences of multiple herbivory on subsequent community members, or the dynamics of a community as a whole.

Next to the identity of inducing herbivores, there are several aspects of multiple herbivory that can play a role in changing insect-plant interactions. The timing of multiple herbivore arrival, the time duration before a plant responds to herbivory and the period that this response lasts, and variation among plant individuals and populations can affect community dynamics.

Insects can arrive on a plant at different moments in the season; early on a relatively undamaged plant, or later in the season on a plant already fully colonized with insects. The timing of insect arrival may play a role in shaping induced plant responses (Gomez *et al.* 2010; Erb *et al.* 2011; Karban 2011; Wang *et al.* 2014) and thus subsequent community dynamics.

Together with the timing of herbivore-induced responses, the duration of these responses determines the effects on future community members. Plant responses to herbivory can occur within minutes to days (Voelckel & Baldwin 2004; Underwood 2012), but we do not know exactly how long induced responses may last, which may be up to days to weeks or even years in perennial plants (Haukioja *et al.* 1985; Underwood 1998; Miller-Pierce & Preisser 2012; Underwood 2012; Stam *et al.*

2014). Insect interactions with a plant thus potentially leave legacies in a plant's phenotype that may influence community dynamics long after the insect has left (Wurst & Ohgushi 2015).

Finally, another source of variation in the dynamics of interaction webs is the genetic background of a plant, for example when plants originate from different populations or closely related species (Gols *et al.* 2008b; Newton *et al.* 2009a; Agrawal *et al.* 2014; Li *et al.* 2014). Plants from different populations can be specific in their responses to herbivory (Johnson & Agrawal 2005; Kroes *et al.* 2016), but knowledge is lacking on population-specific responses to multiple herbivory, and how this specificity translates to community dynamics.

The aim of this thesis project was to study the effects of herbivory by multiple insects on the community dynamics of subsequently arriving community members on a perennial plant. To study how insect-plant dynamics are structured, I conducted field studies with wild perennial cabbage plants, *Brassica oleracea*. I varied the order of arrival and timing between multiple arriving herbivore species as well as the timing of whole-community colonisation on the plant. By following the subsequently naturally colonizing insect community, I gained insight in how the community responds to plant phenotypic changes induced by multiple herbivory. Furthermore, I looked into the variation of plant responses from different populations, as well as how long effects of early-season herbivory to community composition and eventually fitness of this perennial plant lasted. Next to these field studies in which hypotheses could be tested under (semi)natural conditions, greenhouse studies provided more detailed insight in the underlying mechanisms.

Study system

Plant The cabbage plant *Brassica oleracea* L. (Brassicaceae) has been cultivated in many forms for its palatability of leaves, sprouts, stem and flower buds. However, as a wild plant it is less palatable due to many resistance traits against abiotic and biotic stresses (Rubatzky & Yamaguchi 1997; Chen *et al.* 2015). Wild cabbage is a perennial plant growing in coastal areas in Europe (Moyes *et al.* 2000; Wichmann *et al.* 2008), and a large variety of insects ranging from herbivores, predators, parasitoids and pollinators is supported by *Brassica* plants (Newton *et al.* 2009b; Poelman *et al.* 2009; Lucas-Barbosa

Wild cabbage plant, *Brassica olearacea*.

et al. 2014). Resistance traits of cabbage plants consist of morphological traits, such as leaf toughness or a waxy layer, and chemical traits, such as the group of secondary compounds that is known for its resistance character in *Brassica's*, the glucosinolates. Either of the two categories of plant defences can be constitutively present or herbivore-inducible (Schoonhoven *et al.* 2005; Gols *et al.* 2008b; Bukovinszky *et al.* 2009; Poelman *et al.* 2010). *Brassica* plants are specific in their response to feeding by insects from different herbivore feeding guilds (Bidart-Bouzat & Kliebenstein 2011) or even different herbivore species (de Vos *et al.* 2005; Pashalidou *et al.* 2013). The resistance traits can furthermore vary between different populations of wild cabbage plants, even if they originate only tens of kilometres apart (Moyes *et al.* 2000; Gols *et al.* 2008b; Newton *et al.* 2009b). For this thesis, I used plants originating from the Southern English coast, at Kimmeridge (KIM, 50°36'N, 2°07'W), Old Harry (OH, 50°38'N, 1°55'W) and Winspit (WIN, 50°35'N, 2°02'W) (Gols *et al.* 2008b; Newton *et al.* 2010). As a perennial plant, wild cabbage plants have one or several separate vegetative and reproductive growth seasons, producing large amounts of flowers and seeds from their second growth season onwards.

Inducing herbivores Three species have been used to induce plant responses during this thesis project: the phloem-feeding aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae), the leaf-chewing larvae of the moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), and the leaf-chewing larvae of the butterfly *Pieris rapae* L. (Lepidoptera: Pieridae). All three species are specialists on plants of the Brassicaceae family and are known to occur on wild *B. oleracea* plants (Moyes *et al.* 2000). However, they differ in several traits, such as feeding guild, and in the responses they induce in plants upon feeding (Agrawal 2000; Poelman *et al.* 2008a; Ponzio *et al.* 2016). All three species can occur from early in the plant growth season onwards, although their timing of arrival on individual plants is not necessarily fixed (Poelman *et al.* 2009; Poelman *et al.* 2010). The abundance of the

Larva of the moth *Plutella xylostella* (left) and adults and nymphs of the aphid *Brevicoryne brassicae*.

Larva of the butterfly *Pieris rapae*.

B. brassicae population often slowly increases during the season with a high mid-season peak, while both caterpillars have several generations per season (Poelman *et al.* 2008a; Gols *et al.* 2011).

Responding community There are many insect and other invertebrate species that can occur on wild *B. oleracea* throughout its growing season (see Supplementary Table 1 in Chapter 4 for a list of species encountered during two seasons of field research for this thesis). In this thesis, the generalist caterpillar *Mamestra brassicae* L. (Lepidoptera: Noctuidae) in particular has been used as a responding species to herbivore-induced or control plant phenotypes. Generalist herbivores have a wide range of host plants from different families, and are hypothesized to be less adapted to deal with specific plant resistance traits (Schoonhoven *et al.* 2005; Ali & Agrawal 2012). Therefore it is an ideal species for bioassays on herbivore-induced plants to assess the consequences of herbivory to other community members. Not only individual herbivores but also the arthropod community associated to wild *B. oleracea* as a whole can be affected by herbivore-induced plant changes (Li *et al.* 2014; Poelman *et al.* 2014). The occurrence and population dynamics of arthropods associated to wild cabbage may vary within and across seasons and depend on the induced phenotype of the plant (Newton *et al.* 2009a; Newton *et al.* 2010; Li *et al.* 2016). Hence, in three chapters of this thesis the whole arthropod community occurring on individual plants was monitored throughout the season to elucidate effects of previous insect-plant interactions.

Larva of the mothe *Mamestra brassicae*.

Thesis outline

Chapter 2 provides a literature review on how plants interact with multiple herbivores, and how this affects several aspects of community dynamics. A plant phenotype induced by the combination of multiple herbivores at the same time or in sequences may be very different from a plant phenotype induced by single herbivores, due to non-additive interactions or even facilitation between herbivores. Furthermore, a broad context is given for multiple herbivore-plant interactions ranging from a community perspective (plant plasticity affecting dynamics throughout the interaction web), to the individual plant-herbivore level (enhancement or suppression of insect performance), and all the way down to plant cell level (regulation of molecular processes that lead to plant responses).

Each of these processes plays a role at different time scales, in years or weeks or minutes.

Chapter 3 investigates how an herbivore is affected by the timing of different insect herbivore species that had arrived previously on the same plant. As the plant response to herbivory may take some time to be expressed, and may differ for different herbivore species, the timing of arrival on a plant between two herbivore species may change the resulting plant phenotype. In the greenhouse, both the order of two insects, an aphid (*B. brassicae*) and a caterpillar (*P. xylostella*), and the time between the arrival of the two was varied. After they had been allowed to feed on the plant, the preference of a generalist caterpillar (*M. brassicae*) for these damaged plants was tested. Also the performance of this subsequent herbivore on the herbivore-induced plants was assessed.

The question of how the order of two herbivores arriving on a plant affects insects that arrive later on that same plant was brought to the field in **chapter 4**. In nature, insects colonize plants at different moments in time, so that who comes first may differ per individual plant or per year. A field experiment was conducted in two years to assess the insect community composition development throughout the season, affected by the order of induction by two early season herbivores: aphids first or caterpillars first. Furthermore, the specificity of two wild cabbage populations mediating interactions of early season herbivores to the later season community was investigated.

To better understand the mechanism of plant responses to two herbivore species feeding simultaneously on a plant and how this affects other herbivores, a more detailed investigation of multi-herbivore induced plant responses is made in **chapter 5**. Herbivores from different feeding guilds can induce different plant responses. The patterns of cross-talk between different plant biochemical pathways may differ when herbivores feed simultaneously, resulting in a different plant phenotype than when each of the herbivores feeds alone. In a greenhouse setting, gene expression and plant hormone levels were measured at several points in time after aphids (*B. brassicae*) and/or caterpillars (*P. xylostella*) had eaten from the plants alone or simultaneously. Subsequently, the performance of a generalist caterpillar (*M. brassicae*) feeding from these herbivore-induced plants was measured to evaluate how a change in plant phenotype by multi-herbivory influences a later colonizer.

The goal of **chapter 6** was to test how long plant-mediated interactions persist in the community, and whether it matters for plant fitness. Herbivores can interact with other herbivores that are separated in time, *via* induced plant responses

that mediate the interaction. An important question is whether such legacies also last across a winter without herbivory? In a two-year-long field experiment, the development of the community composition on perennial *B. oleracea* plants was followed, after the plants had been induced by aphid or caterpillar feeding at the beginning of either year. To test legacy effects of the herbivore or carnivore community onto plant fitness within or across years, seeds of the plants were collected at the end of the second season, when the plants had flowered for the first time. Plant performance traits were measured to assess whether they could explain the community interactions.

In **chapter 7** the importance of the arthropod community history and plant ontogeny for shaping community development was assessed. The interaction web of an insect-plant system depends on the insects that were previously present on the plant, but also on the growth-state-dependent responsiveness of the plant to insect herbivory (plant ontogenetic variation in defence allocation). To assess whether these processes interact at the moment when a new herbivore arrives on a plant, a field experiment was carried out. Either the colonisation of the insect community was excluded for a shorter or longer time, or a caterpillar (*P. rapae*) was placed on the plant at different points in time during the season, or both. The observed subsequent community development was modelled to assess additive or non-additive effects of either process.

Finally, in **chapter 8** all previous chapters are integrated and the findings are discussed in an eco-evolutionary context. I discuss the importance of the timing of multiple inducing herbivores, as well as timing of plant responses as measured in community dynamics, for shaping the resulting community. These aspects can have important consequences for plant interactions with multiple herbivores, because they affect the variability of both plant responses to insects and insect responses to plant phenotype. Perspectives on future research directions of plant interactions with the whole associated community are highlighted at the end of the chapter.

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Chapter 2

Plant interactions with multiple insect herbivores: from community to genes

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Abstract

Every plant is a member of a complex insect community that consists of tens to hundreds of species that belong to different trophic levels. The dynamics of this community are critically influenced by the plant, which mediates interactions between community members that can occur on the plant simultaneously or at different times. Herbivory results in changes in the plant's morphological or chemical phenotype that affect interactions with subsequently arriving herbivores. Changes in the plant's phenotype are mediated by molecular processes such as phytohormonal signaling networks and transcriptomic rearrangements that are initiated by oral secretions of the herbivore. Processes at different levels of biological complexity occur at timescales ranging from minutes to years. In this review, we address plant-mediated interactions with multiple species of the associated insect community and their effects on community dynamics, and link these to the mechanistic effects that multiple attacks have on plant phenotypes.

Keywords

Phenotypic plasticity, trait-mediated interaction networks, phytohormones, systems biology, species interactions

Introduction

Plants are members of biodiverse communities consisting of a microbiome (Mendes *et al.* 2011) and a macrobiome (Whitham *et al.* 2006; Dicke & Baldwin 2010). The microbiome consists of, e.g., symbiotic microorganisms such as mycorrhizae, endophytes, and nitrogen-fixing bacteria; plant pathogenic microorganisms; and their antagonists (Hartley & Gange 2009; Pineda *et al.* 2010). The macrobiome consists of herbivores and their natural enemies, such as predators and parasitoids, as well as pollinators (Figure 1). For each plant species, the combined macrobiome and microbiome can easily comprise several hundred species that belong to different trophic levels (Harvey *et al.* 2009) (Figure 1). Moreover, each individual plant is surrounded by a range of other plant individuals of the same or different species, which compete for light and nutrients (Cerrudo *et al.* 2012) and share members of the microbiome and macrobiome.

Understanding the functioning of this complex of interacting species requires studies of their population dynamics in space and time and the underlying trophic and informational mechanisms. In this review, we focus on plants and their associated insect communities. Insects are the most speciose group of organisms, comprising an estimated 6 million species, of which 50% are herbivorous, and the 300,000 plant species represent the group of organisms with the largest biomass (Schoonhoven *et al.* 2005). Thus, communities of insects and plants make up a significant proportion of life on Earth.

Feeding by herbivorous insects influences the phenotype of their food plant (Kessler & Baldwin 2002; Dicke & Baldwin 2010; Mithöfer & Boland 2012), which consequently influences the interactions of the plant with its associated community (Ohgushi 2005; Utsumi *et al.* 2010; Poelman *et al.* 2011). Such herbivore-induced effects may last throughout the growing season of the plant or for several years (Haukioja 1980; Thaler *et al.* 2001; Johnson & Agrawal 2007; Poelman *et al.* 2008a).

Research on plant–insect interactions has addressed mainly the effects of interactions between one plant and one insect species. This has yielded important knowledge on how insects find and select their host plants and deal with plant defenses (Schoonhoven *et al.* 2005) as well as how herbivory modifies plant phenotypes (Kessler & Baldwin 2002; Dicke & Baldwin 2010; Mithöfer & Boland 2012).

However, because plants are members of complex communities, interactions with multiple attackers are the rule rather than an exception (Ohgushi 2005; Dicke *et al.* 2009; Utsumi *et al.* 2010). Moreover, attacks by different organisms interact at different levels of biological organization, ranging from the subcellular level (Pieterse *et al.* 2012) to the individual (Kaplan & Denno 2007) and community levels (Poelman *et al.* 2008b). Studies on the interactions between plants and their associated insect communities have received increasing attention and have addressed effects at the levels of gene expression, phytohormonal crosstalk, metabolomic changes, species

interactions, and community dynamics. The current focus in the field of plant–insect interactions is on connecting different levels of biological organization (Keurentjes *et al.* 2011; Baldwin 2012), which is already challenging for individual plant–insect interactions and certainly so for multiple attacks on a single plant, and therefore requires a multidisciplinary approach.

In this review, we address the effects of multiple attacks on plants at different levels of biological organization in an integrative way. Although plants are members of plant communities that comprise individuals from different species, we limit this review to individual plants, and particularly interactions with multiple insect species aboveground. We also limit the review to plants in the vegetative stage, because most information is available for this plant stage. We conclude with an outlook on the future of this rapidly developing, multidisciplinary field.

Plants affect insect community composition and dynamics

The composition and dynamics of the insect community that interacts with a plant are influenced by plant traits such as chemistry, physiology, and morphology (Ohgushi 2005; Johnson *et al.* 2006; Whitham *et al.* 2006; Bukovinszky *et al.* 2008; Harvey *et al.* 2011), which have a genetic basis. Thus, the genotype of a plant and, consequently, the expressed plant phenotype affect insect community members that interact with the plant and shape the composition of the community (Whitham *et al.* 2006; Whitham *et al.* 2012). The insect community together with the plant phenotype gives rise to the community phenotype, and plant individuals with similar traits tend to support similar insect communities (Johnson *et al.* 2006; Whitham *et al.* 2006; Keith *et al.* 2010). A plant's genotype can have size- and density-mediated effects on the associated insect community. For example, plant traits may affect the sizes of herbivores and therefore the sizes of parasitoids (Figure 1d, e) that develop in the herbivores, and even the sizes of hyperparasitoids (Figure 1f, g) that develop in those parasitoids that develop in the herbivores (Bukovinszky *et al.* 2008). Moreover, plant genotype may affect the density of herbivores, parasitoids, and hyperparasitoids as well as the composition of the herbivore, parasitoid, and hyperparasitoid communities on these plants (Bukovinszky *et al.* 2008).

Chemical plant traits are well known to be crucial components of the plant phenotype that mediate plant–insect interactions (Schoonhoven *et al.* 2005). Genotypic variation affects plant chemical traits, which has consequences for species interactions and community dynamics. An example of an extensively studied plant chemical trait that affects insect community composition is condensed tannin concentration, especially in tree species (Whitham *et al.* 2006; Schweitzer *et al.* 2008). Tannins are known to negatively influence herbivorous insects (Schoonhoven *et al.* 2005), and the concentration of tannins in poplar trees indeed affects the composition of insect communities (Whitham *et al.* 2006). Tannins usually reduce insect

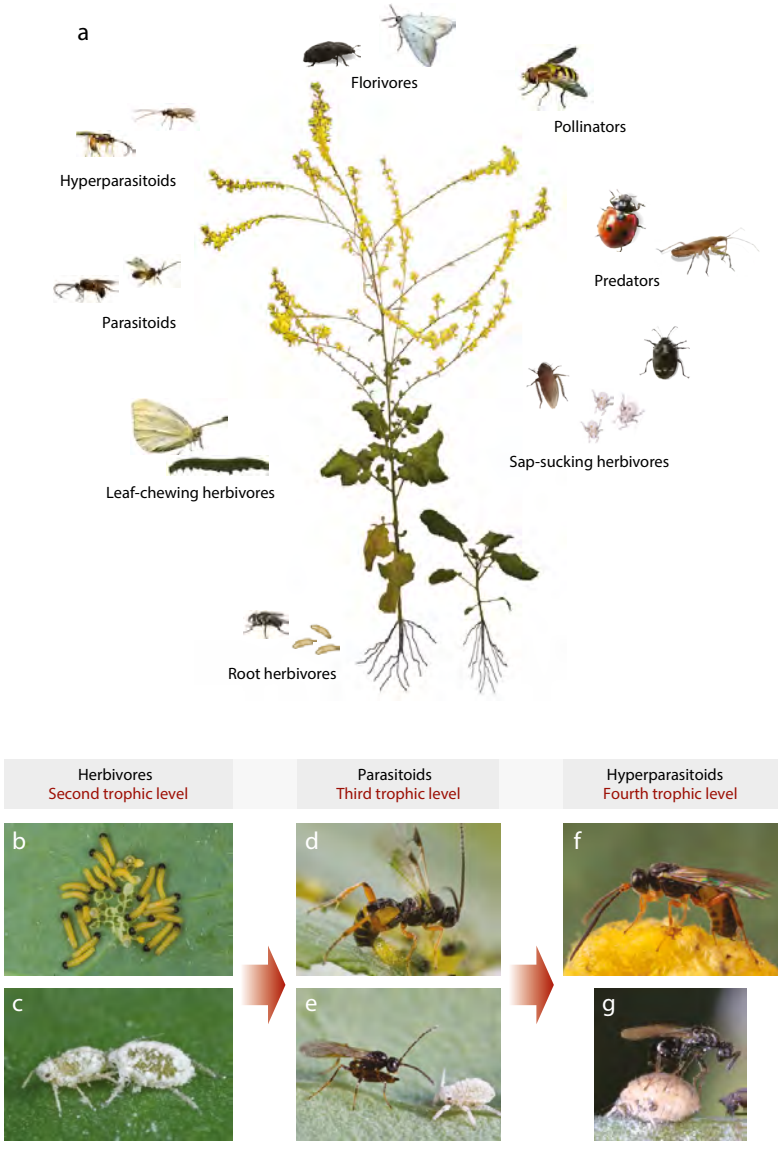

Figure 1. Insect community associated with *Brassica nigra* (black mustard) plants and specific representatives of some members of this community. Community overview (a). Biting/chewing herbivores (*Pieris brassicae* caterpillars) (b). Piercing/sucking herbivores (*Brevicoryne brassicae* aphids) (c). A parasitic wasp (*Cotesia glomerata*) attacking *P. brassicae* caterpillars (d). A parasitic wasp (*Diaeretiella rapae*) attacking a *B. brassicae* aphid (e). A hyperparasitoid (*Lysibia nana*) parasitizing pupae of the parasitoid *Cotesia glomerata* (f). A hyperparasitoid (*Asaphes* sp.) parasitizing a parasitoid that itself has parasitized a *B. brassicae* aphid (g). Photo credits: Tibor Bukovinszky (panels b, c, and g), Hans Smid (panel d), and Nina Fatouros (panels e and f) (http://www.bugsinthepicture.com).

growth rate (Schoonhoven *et al.* 2005), although tannins may also positively affect insect performance or preference; the effects of tannins are likely dependent on species, tissue, and context and influenced by other chemical constituents of plant tissue (Schweitzer *et al.* 2008). Tannins can also affect community members indirectly through a negative effect on nitrogen mineralization, which subsequently feeds back to root production and consequently to the nutritional value of the tree (Whitham *et al.* 2006), with long-term effects on herbivorous insects (Schweitzer *et al.* 2008). Thus, condensed tannin levels affect community phenotypes (Whitham *et al.* 2006).

In annual or perennial nonwoody plant species, family-specific secondary chemistry can shape the community phenotype. For instance, glucosinolates, which are characteristic secondary metabolites of plants in the Brassicaceae family, have important effects on insect community composition (Hopkins *et al.* 2009; Newton *et al.* 2009a; Poelman *et al.* 2009). The quality and quantity of these compounds are known to deter generalist insect species or hamper their development, whereas they may be used for feeding and or as oviposition stimulants by specialist species (Hopkins *et al.* 2009). Differences in glucosinolate composition among *Brassica oleracea* cultivars resulted in large differences in herbivore community dynamics (Poelman *et al.* 2009) that resemble community differences observed in natural populations of *B. oleracea* plants that differ in their chemical profiles (Newton *et al.* 2009a).

In addition to plant secondary chemistry, many other plant traits can affect insects. These traits include plant biomass and architecture (Andow 1991; Johnson & Agrawal 2005; Ohgushi 2005; Schoonhoven *et al.* 2005), leaf morphology (Barbour *et al.* 2009), trichome density (Johnson 2008), and plant nutritional value in terms of water and nitrogen content (Scriber & Slansky 1981; Johnson 2008).

Consequences of plant traits for insect herbivores

To understand how a plant's genotype affects community composition and dynamics, knowledge of the underlying mechanisms is important. Individual plant traits have different effects on different community members.

Among insect herbivores contrasting dietary categories are observed. Generalist species feed on plants belonging to phytochemically unrelated families, whereas specialist species utilize only plant species within a single family or a single genus (Schoonhoven *et al.* 2005; Ali & Agrawal 2012). Generalist herbivores are usually more sensitive to plant defense compounds, whereas specialist herbivores may use these same compounds as recognition cues (known as token stimuli) (Gols *et al.* 2008a; Gols *et al.* 2008b)**.** Adaptation to plant chemicals specific for certain plant taxa through specialized detoxification or sequestration mechanisms allows specialists to utilize some plants as food and exploit such chemicals for their own defense, whereas generalists are either unable to survive or grow or have a reduced

survival or growth rate on such plants (Hopkins *et al.* 2009; Ali & Agrawal 2012).

Insect herbivores can also be classified based on feeding guilds—e.g., leaf chewers, phloem feeders, leaf miners, root feeders, and gall-inducing insects—which may differ in their responses to plant traits. Whereas leaf chewers often consume whole leaves and thus are exposed to chemicals in all leaf cells, phloem feeders such as aphids specialize on the phloem. Some secondary compounds that react with each other to form a toxic compound only upon rupture of multiple cells by chewing are thus circumvented by piercing/sucking phloem feeders (Schoonhoven *et al.* 2005).

Some plant traits are likely to affect all herbivores, whereas others affect only a particular subset, e.g., based on herbivore size. A plant with high leaf toughness will affect many herbivore species, although some species are better able to deal with this than others (Agrawal 2005). In contrast, a high trichome density particularly affects smaller insects that walk in a forest of leaf hairs (Dussourd 1995; Schoonhoven *et al.* 2005), and secondary metabolites particularly affect generalist insects (see above).

Because distinct herbivorous members of a community respond differently to the same plant traits, each trait differentially influences community composition. The many interactions that occur between the various plant traits and the diverse community members, and among herbivore members themselves, potentially increase the complexity of underlying mechanisms that modulate community composition. However, only one or a few so-called foundation species may have a major effect on the community composition (Whitham *et al.* 2006). Keith et al. (2010) proposed that a few plant traits particularly affect one or a few foundation herbivore species, which subsequently affect the community. This suggests that effects of plant traits might be passed on not only to single species but also to a whole chain of interacting species.

Consequences of plant traits for insect carnivores

The discussion above considered mainly plant–herbivore interactions, but plant traits also affect organisms at higher trophic levels, such as predators or parasitoids of herbivores as well as carnivorous insects at even higher trophic levels (Price *et al.* 1980; Bukovinszky *et al.* 2008; Heil 2008; Harvey *et al.* 2009; Dicke & Baldwin 2010; McCormick *et al.* 2012; Poelman *et al.* 2012). Plant traits can directly affect the natural enemies of herbivores, for example, by providing shelter (Romero & Benson 2005; Schoonhoven *et al.* 2005) or extrafloral nectar as food (Heil *et al.* 2001; Schoonhoven *et al.* 2005). Plant traits can also affect higher trophic levels either directly, through reduced quality of the herbivores (Bukovinszky *et al.* 2008), or indirectly, through exposure to phytochemicals ingested by the herbivore (Gols & Harvey 2009). Such indirect interactions with herbivores as a mediator between plant traits and predators or parasitoids can have large effects on the community composition at the second, third, and even higher trophic levels (Whitham *et al.*

2006; Bukovinszky *et al.* 2008; Harvey *et al.* 2009; Smith *et al.* 2011; Poelman *et al.* 2012). For example, evening primrose genotype affected aphid population growth rate directly as well as indirectly through effects on the abundance of aphid-tending ants and the diversity of predators (Johnson 2008). Similar results were found for parasitoids of caterpillars feeding on genetically different willows (Fritz *et al.* 1997) or cabbage plants (Bukovinszky *et al.* 2008; Harvey *et al.* 2011). The adaptation of herbivores to specific plant traits might even affect the evolution of members of higher trophic levels, leading to specialization of parasitoids on herbivores that are adapted to plant traits (Stireman *et al.* 2006). Plant effects on the composition of the herbivore community can also affect the foraging behavior of carnivores. For example, the foraging success of parasitoids that search for hosts is affected by the presence and identity of additional, nonhost herbivores on the plant (de Rijk *et al.* 2013).

Plant traits may also interfere with the performance of carnivorous insects, thereby providing herbivores with enemy-free space. For instance, in pea plants, a leafless mutation that affects plant architecture hampers the foraging behavior of lady beetles, which results in enhanced population growth of aphids (Kareiva & Sahakian 1990).

Top-down effects

The bottom-up effects of plant traits on higher trophic levels (herbivores and their natural enemies) discussed above may be strong predictors of community composition (Kos *et al.* 2011), but top-down effects of natural enemies on herbivores can have important effects on community composition as well (Hunter & Price 1992). Predators or parasitoids consume their hosts partly or completely and therefore constrain the population density of herbivores attacking a plant. Parasitoids can exert significant top-down control of herbivore populations (van Veen *et al.* 2005), and their activities can influence competition between herbivore species (van Veen *et al.* 2006). Interestingly, the elimination of a single parasitoid species from a small community resulted in the extinction of other parasitoid species that were four trophic links away (Sanders *et al.* 2013). This included effects mediated through herbivores. Thus, top-down effects can be sequentially linked to bottom-up effects (Kareiva & Sahakian 1990; Sanders *et al.* 2013).

Insects at the third trophic level do not always have a negative effect on herbivore species: For example, ants may tend aphids and thus protect them from their natural enemies (Johnson 2008). Although the ants have a positive effect on the aphids, they may also prey on other herbivores that share the plant with the aphids (Vrieling *et al.* 1991).

In conclusion, plant traits influence members of the associated insect community at different trophic levels, and species at higher trophic levels affect the dynamics of species at lower trophic levels. Many of these plant traits are constitutively

expressed. Moreover, community dynamics are also influenced in important ways by the fact that insects modify plant phenotype. The modification of plant phenotype by herbivore attack is the focus of the remainder of this article. We address the effects of phenotypic modification by herbivory on insects at different trophic levels, the molecular mechanisms underlying the phenotypic modification, and how different herbivore species that attack the same plant interfere with one another's effects on the plant's phenotype. Finally, we address the effects of herbivore-induced modification of plant phenotype on community dynamics.

Herbivore-induced change in plant traits

Herbivory or egg deposition by herbivores alters plant phenotype through changes in the production of primary and secondary metabolites, morphological traits, and architecture (Kessler & Baldwin 2002; Howe & Jander 2008; Dicke & Baldwin 2010; Hilker & Meiners 2010; Mithöfer & Boland 2012) (Figure 2). Such herbivore-induced plant responses may affect the behavior and growth of the initial attacker and may also influence host-plant suitability for other herbivores, even when these are temporally or spatially separated, thus mediating interspecific competition between insect herbivores (Denno *et al.* 1995; Ohgushi 2005; Ohgushi 2008) (Figure 2). Furthermore, the effects of herbivore-induced alterations in plant phenotype are to some extent specific to the attacking herbivores, and they may affect subsequent herbivores either positively or negatively, depending on the characteristics of the responding herbivore species (Kaplan & Denno 2007). For example, spider-mite infestation of cotton plants increased resistance against conspecific mites and whiteflies but also enhanced susceptibility to aphids (Agrawal *et al.* 2000). Willow infestation by leaf rollers enhanced the abundance of aphids and ants but also reduced the abundance of leaf beetles (Ohgushi 2005).

Herbivore-induced resistance to herbivores

Herbivore-induced resistance of plants to herbivores is a common phenomenon and has been described for many insect herbivores of various feeding guilds (Karban & Baldwin 1997). For example, through induced changes in plant phenotype, feeding by lepidopteran larvae prolongs immature development of other lepidopteran species that colonize a common host plant later in the season (Agrawal 2000; Poelman *et al.* 2008a). A meta-analysis of genetic correlations between plant levels of resistance to multiple enemies revealed positive correlations when the compared species were both generalist herbivores or when they were both specialist herbivores (Leimu & Koricheva 2006). It also revealed significant positive genetic correlations for plant resistance to herbivores from different feeding guilds, such as miners and gall inducers, miners and leaf folders, and gall inducers and leaf folders (Leimu & Koricheva 2006). In pairwise comparisons of interactions between

herbivores belonging to different feeding guilds, the lowest genetic correlation was recorded for mechanisms of plant resistance to phloem-feeding and leaf-chewing herbivores (Leimu & Koricheva 2006).

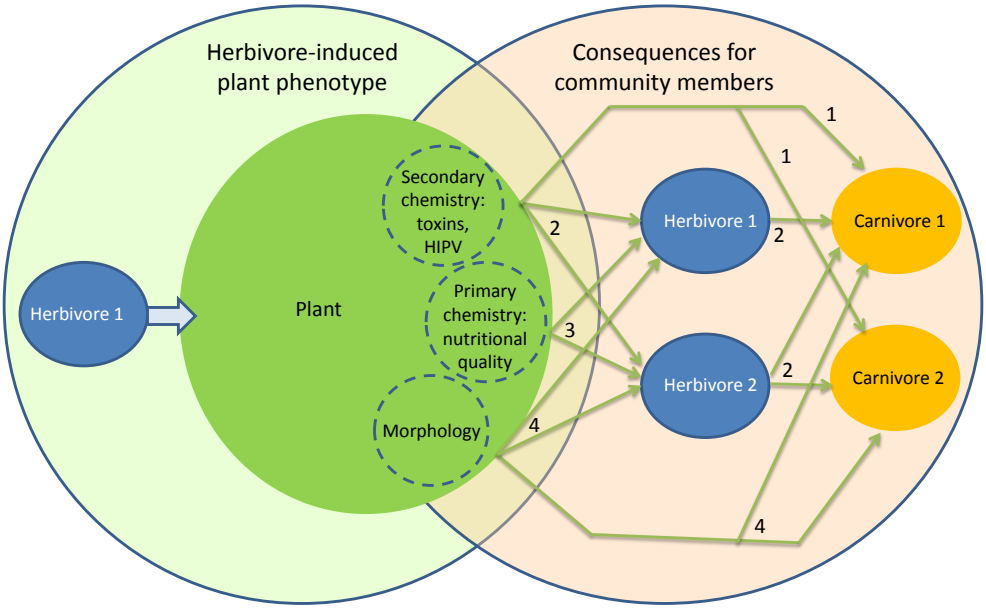

Figure 2. Components of plant phenotypic plasticity in response to herbivore attack and the interactions of these components with other members of the insect community: (1) herbivore-induced plant volatiles that attract carnivorous insects (HIPV); (2) secondary plant metabolites such as toxins and digestibility reducers that affect the performance of herbivores and through herbivores may affect their carnivorous enemies; (3) primary plant metabolites that are used as nutrients by herbivores; (4) morphological characteristics such as trichomes and cuticular wax layers that affect the performance of herbivorous insects and the behavior of their carnivorous enemies.

Herbivore-induced susceptibility to herbivores

Herbivore-induced susceptibility seems to be less common than herbivore-induced resistance (Leimu & Koricheva 2006), and in half of the reported cases it involved interactions between piercing/sucking and biting/chewing herbivores (Denno *et al.* 1995). Yet 20–40% of the total number of interactions within the herbivore community associated with willow and goldenrod were facilitative (Ohgushi 2008). Most facilitative interactions were asymmetric, with only one species gaining an advantage (Denno *et al.* 1995; Kaplan & Denno 2007).

Different mechanisms may underlie facilitation among different herbivore species. For example, the facilitative interaction between spittlebugs and leaf rollers that was observed on willow was caused by compensatory shoot growth in response to

spittlebug infestation; leaf rollers prefer leaves on the new shoots (Ohgushi 2005). A stem-boring moth induced susceptibility in willow to a specialist leaf beetle by causing young shoot growth (Utsumi & Ohgushi 2008). Herbivory by leaf rollers on oak provided shelter and better feeding sites for aphids (Karban *et al.* 1997; Karban & Agrawal 2002), and herbivory by aphids interfered with induced defense signaling against caterpillars (Soler *et al.* 2012a).

Herbivore-induced plant responses and carnivorous insects

Herbivore-induced changes in plant secondary chemistry play an important role in habitat and host location of carnivorous insects, mainly via the production of volatiles in response to feeding by their prey or hosts (D'Alessandro & Turlings 2006; Dicke & Baldwin 2010; McCormick *et al.* 2012) (Figure 2). These herbivore-induced plant volatiles attract the carnivorous enemies of herbivores to plants infested with their herbivorous victim. Moreover, even hyperparasitoids at the fourth trophic level may exploit herbivore-induced plant volatiles to find their parasitoid host that feeds within an herbivorous insect (Poelman *et al.* 2012). However, specific volatile chemicals or mixtures of chemicals may also repel carnivorous insects (Snoeren *et al.* 2010; Webster *et al.* 2010; Braasch *et al.* 2012). Volatile-mediated foraging behavior of carnivores is more difficult to predict when multiple herbivores attack the same host plant (Shiojiri *et al.* 2001; Dicke & Baldwin 2010; Ponzio *et al.* 2013). When nonhost herbivores share the same plant individual with hosts, changes in the induced volatile blend can interfere with host location by foraging carnivorous insects (Dicke *et al.* 2009; de Rijk *et al.* 2013).

Nonvolatile plant chemistry may also mediate the effects of herbivore-induced changes in plant phenotype on carnivores (reviewed in Gols and Harvey (2009) and Ode (2006)). Herbivore-induced changes in plant chemistry may prolong herbivore development and consequently extend the exposure period of the herbivore to its enemies (Benrey & Denno 1997). Moreover, some specialist herbivores are able to sequester plant secondary metabolites and exploit these defenses for their own protection from natural enemies (Kazana *et al.* 2007; Müller 2009) (Figure 2). Herbivore-induced plants may also influence immune responses of herbivores to parasitoids (Bukovinszky *et al.* 2009). *Pieris rapae* caterpillars that developed on plants previously damaged by *Pieris brassicae* caterpillars had a reduced ability to encapsulate parasitoid eggs compared with those reared on undamaged plants (Bukovinszky *et al.* 2009). It is remarkable that herbivory resulted in inferior performance and immune response of the subsequent caterpillars and enhanced their susceptibility to parasitism. However, suppressed performance of host caterpillars on induced plants may also inhibit parasitoid performance through reduced host nutrient availability (Ode 2006). Generalist parasitoids tend to be more susceptible to inducible plant metabolites than specialist parasitoids are (Gols *et al.* 2008b; Bukovinszky *et al.* 2012).

In conclusion, herbivory alters plant phenotype, which has consequences for the interactions of the plant with herbivorous and carnivorous insects (Figure 2). In the next section, we address the molecular mechanisms underlying the modification of plant phenotype by herbivory and how different herbivores feeding on the same plant affect one another's modifications.

Molecular mechanisms underlying plant phenotypic plasticity under single and multiple attacks

The past decade has brought significant advances in the mechanistic understanding at the (sub)cellular level of induced plant responses that underlie plant–insect interactions (Kessler & Baldwin 2002; Maffei *et al.* 2007; Felton & Tumlinson 2008; Howe & Jander 2008; Wu & Baldwin 2010; Bonaventure *et al.* 2011; Maffei *et al.* 2012; Mithöfer & Boland 2012; Reymond 2013). This relates to the recognition of attackers and the induction of signal transduction pathways, which is followed by transcriptomic changes and the induction of biosynthetic pathways leading to changes in plant phenotype. Most of this research has focused on interactions between a plant and one attacker, but over the past decade, studies of the interactive effects of the combined infestation of a plant by two attackers have been initiated (Kessler & Baldwin 2004; Voelckel & Baldwin 2004; Dicke *et al.* 2009; Rodriguez-Saona *et al.* 2010; Thaler *et al.* 2012a; Zhang *et al.* 2013).

Signal transduction pathways

Herbivorous insects produce oral secretions containing compounds that elicit plant responses (Bonaventure *et al.* 2011). The chemical nature of the active compounds is remarkably diverse and includes small organic compounds such as benzyl cyanide, fatty acid–amino acid conjugates, and proteins such as β-glucosidase (Maffei *et al.* 2012). The initial step in the elicitation process occurs with considerable specificity for the plant–insect combination studied. The recognition of herbivore elicitors by plant receptors initiates a cascade of responses, including changes in plasma membrane potential and activation of networks of kinases and phytohormones (Maffei *et al.* 2007). More recently, it has become apparent that insects may also produce so-called effectors that function to suppress the elicitor-triggered plant defense response, such as glucose oxidase in the interaction between *Helicoverpa zea* caterpillars and tobacco (Felton & Tumlinson 2008; Maffei *et al.* 2012). Studies elucidating the regulatory mechanisms underpinning plant defense responses to insect herbivore attack have identified the central role of phytohormones. Three major plant hormones—jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Figure 3a)—function in a complex regulatory network that is essential in herbivore-induced defense responses. Other hormones, such as cytokinins, abscisic acid, gibberellins, and auxin, likely also play a role in herbivore-induced defense signaling

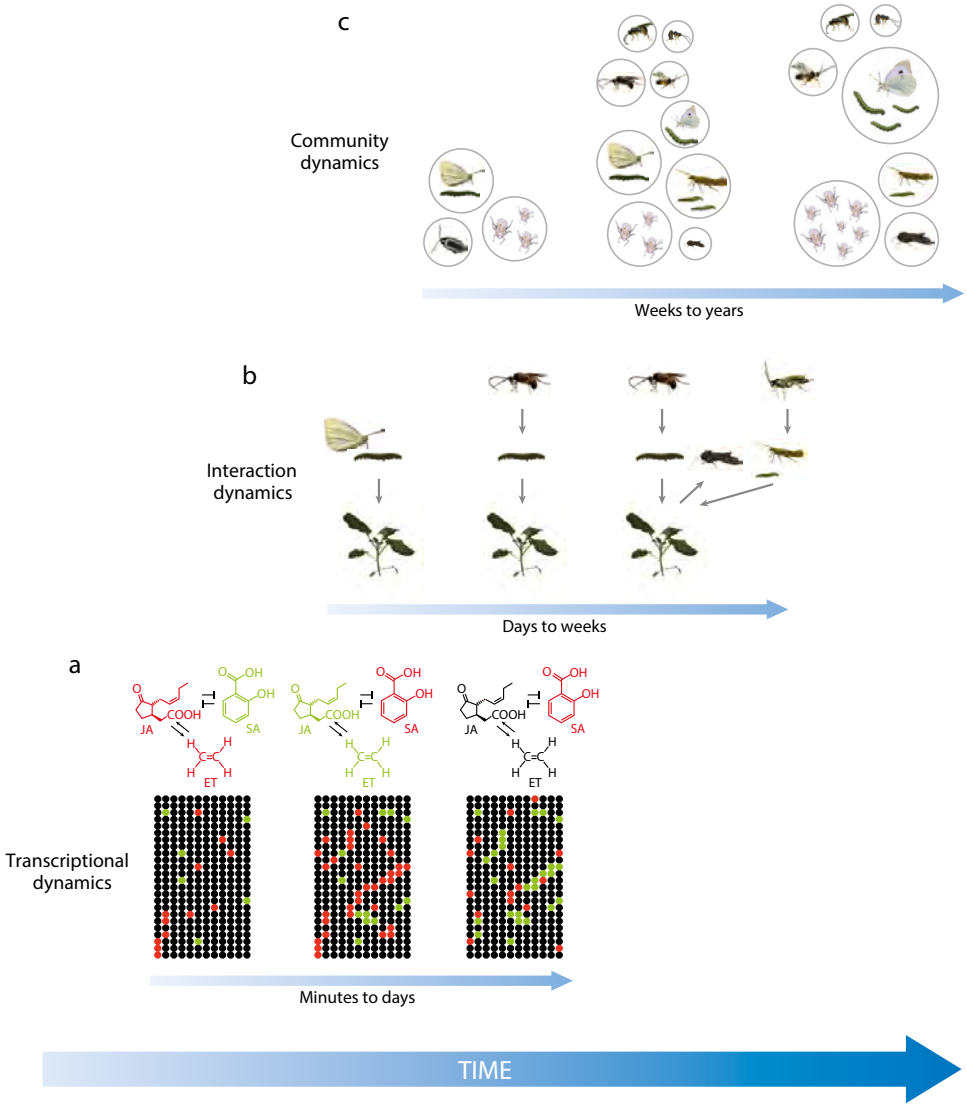

Figure 3. Schematic representation of dynamics at different levels of biological integration, each with its own timescale. Phytohormonal and transcriptional responses to herbivory at a scale of minutes to days (a). The tissue concentrations of the phytohormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), which are involved in defense responses, change dynamically and exhibit crosstalk (arrows); their molecular structures are shown in red when increasing, in green when decreasing, and in black when constant. The dots represent genes in a heat map of gene transcription and are colored red when increasing, green when decreasing, and black when constant. Interactions among individual insects at different trophic levels at a scale of days to weeks (b). Community dynamics at a scale of weeks to years (c).

(Erb *et al.* 2012; Pieterse *et al.* 2012).

It is well documented that chewing herbivores and sap feeders induce different plant signaling pathways involving the three major phytohormones, JA, SA, and ET (Pieterse *et al.* 2012). Much less is known about signaling pathways involved in resistance against insects of other feeding guilds, such as leaf miners, stem borers, leaf folders, and gall-inducing herbivores. SA and ET signaling pathways are involved in the resistance of rice plants to the leaf folder *Cnaphalocrocis medinalis* (Wang *et al.* 2011). Some leaf miners and gall-inducing insects modulate plant cytokinin levels, probably to manipulate the source–sink status of the infected tissues (reviewed in Erb et al. (2012) and Giron et al. (2013)). Feeding by gall-inducing insects increases auxin level but does not change JA level (Tooker & De Moraes 2008; Erb *et al.* 2012). Insect eggs have been reported to induce plant responses via the SA signaling pathway (Reymond 2013).

The salicylic acid pathway

SA regulates induced plant responses against phloem-feeding insects and biotrophic pathogens (Glazebrook 2005; Pieterse *et al.* 2012). In response to phloem-sucking insects, SA can be synthesized from chorismate through the isochorismate pathway (Wildermuth *et al.* 2001) and the phenylalanine ammonium lyase pathway (Dempsey *et al.* 2011). Accumulation of SA leads to the translocation of the positive regulatory protein nonexpressor of pathogenesis-related genes 1 (NPR1) to the nucleus. Regulation of the expression of SA-responsive genes occurs downstream of NPR1, which interacts with TGA-type transcription factors and additionally targets *WRKY* transcription factor genes (Wang *et al.* 2006). This results in the activation of defense gene expression and the production of pathogenesis-related (PR) proteins (Durrant & Dong 2004).

The jasmonic acid/ethylene pathway

JA is an important regulator of defense responses against chewing insects, necrotrophic pathogens, and cell content feeders such as spider mites and thrips (de Vos *et al.* 2005; Glazebrook 2005; Kant *et al.* 2008; Pieterse *et al.* 2012). Upon herbivory, JA is produced via the octadecanoid pathway. In *Arabidopsis*, the enzyme jasmonoyl isoleucine conjugate synthase 1 (JAR1) activates JA by conjugating it to the amino acid isoleucine (Ile) to form JA-Ile (Staswick & Tiryaki 2004). Binding of JA-Ile to the F-box protein coronatine-insensitive 1 (COI1) mediates the degradation of jasmonate ZIM domain (JAZ) repressor proteins (Thines *et al.* 2007). These proteins repress JA signaling by binding transcriptional activators such as MYC2. When the repression of JAZ proteins is lifted, JA-responsive genes are activated, including genes encoding JAZ proteins, resulting in a negative-feedback loop (Memelink 2009). Two branches have been identified within the JA signaling pathway that

act antagonistically (Pieterse *et al.* 2009; Pieterse *et al.* 2012). The MYC2 branch positively regulates the expression of wound-inducible JA-responsive marker genes such as *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) and *LIPOXYGENASE 2* (*LOX2*). In the ethylene response factor (ERF) branch of the JA pathway, JA and ET synergistically induce the expression of JA/ET-responsive transcription factors, including ERF1 and octadecanoid-responsive *Arabidopsis* 59 (ORA59), which positively regulate JA/ET-responsive genes such as *plant defensin 1.2* (*PDF1.2*) (Lorenzo *et al.* 2004; Dombrecht *et al.* 2007). The ERF branch is especially involved in induced defense against necrotrophic pathogens, whereas the MYC2 branch mediates defense against herbivorous insects (Pieterse *et al.* 2012).

Phytohormonal crosstalk and its molecular mechanisms

When a plant faces multiple herbivore attack, crosstalk may occur between the induced signaling pathways, with consequences for induced defense responses. Crosstalk between signaling pathways allows the plant to fine-tune its defense response to the specific attacker (Pieterse *et al.* 2012). For instance, induced defense is regulated through interconnection of the JA, SA, and ET signal transduction pathways (Pieterse *et al.* 2012). Crosstalk between JA and SA signaling is mutually antagonistic, resulting in the prioritization of SA-dependent defense responses over JA-dependent responses or vice versa (Pieterse *et al.* 2012; Thaler *et al.* 2012b). Molecular players that modulate this JA-SA crosstalk include mitogen-activated protein kinases (MAPKs), WRKY transcription factors, the regulatory protein NPR1, and other phytohormones (Pieterse *et al.* 2012). NPR1 is a major regulator of JA-SA crosstalk in *Arabidopsis*, and its effect is mediated by ET, which may have been induced by both biotic and abiotic stresses (Leon-Reyes *et al.* 2009). In contrast to JA-SA crosstalk, JA- and ET-dependent signaling pathways act synergistically in inducing plant defense responses (Pieterse *et al.* 2009).

Crosstalk between phytohormonal signaling pathways also allows herbivores to manipulate plant defenses for their own benefit (Pieterse & Dicke 2007). Feeding by *Manduca sexta* caterpillars induced an ET burst and suppressed nicotine accumulation in tobacco plants (Kahl *et al.* 2000). It has been hypothesized that by activating the SA signaling pathway, phloem feeders suppress JA-dependent defenses to which phloem feeders are more sensitive (Moran *et al.* 2002; Zarate *et al.* 2007). Several recent studies have supported the interference of SA with JA-inducible defenses against chewing insects (Zhang *et al.* 2009; Soler *et al.* 2012a; Thaler *et al.* 2012a; Zhang *et al.* 2013), although phloem-feeding insects do not in all cases interfere with the defenses induced by chewing herbivores (Erb *et al.* 2010), which may be due to density effects or to differences between species.

Transcriptomic changes in response to individual attackers and multiple attacks

Phytohormonal responses to herbivory result in transcriptional responses that have a high degree of specificity. Transcriptional responses depend on the feeding guild of the attacker and the phytohormonal signal signature that the attacker induces. For instance, attack by single insect species belonging to different feeding guilds resulted in the activation of specific sets of defense-related genes in *Arabidopsis* (de Vos *et al.* 2005). Different species of leaf-chewing herbivores that all induced JA in the plant still induced different transcriptomic changes (Bidart-Bouzat & Kliebenstein 2011). These induced transcriptomic changes also differed from those induced by JA, most likely because each attacker activates more than one phytohormonal pathway. De Vos et al. (2005) hypothesized that the phytohormonal signal signature regulates the specific transcriptomic changes. Aphid feeding affected the expression of a substantially larger number of genes compared with feeding by caterpillars and thrips, and it tends to induce gene sets more similar to those induced by fungal or bacterial pathogens (de Vos *et al.* 2005). In *Nicotiana attenuata*, aphids suppressed more genes than chewing herbivores did, and aphids upregulated the expression of SA-dependent genes and suppressed the expression of JA-mediated genes (Heidel & Baldwin 2004). Similar findings were recorded for the effects of feeding by caterpillars and aphids on tomato (*Solanum lycopersicum*) (Rodriguez-Saona *et al.* 2010).

Transcriptomic changes in response to phloem-feeding insects

Phloem-feeding insects, such as aphids and whiteflies, cause little damage to the plant tissue because they move their stylets in between plant cells on their way to the phloem, briefly puncturing but not killing cells along the way. SA accumulates in plants upon interactions with aphids and whiteflies, whereas activation of JA leads to resistance to phloem-feeding herbivores. Early transcriptional responses of *Arabidopsis* to *Brevicoryne brassicae* aphids were observed after 6 h, at which point a group of WRKY transcription factors were highly expressed. Genes involved in SA-dependent defense had a peak expression after 24 h of infestation. After 12 h of aphid infestation, the number of inducible genes expressed and the intensity of JA-inducible responses had already decreased (Kuśnierczyk *et al.* 2008).

Transcriptomic changes in response to chewing insects

Plants respond to feeding by chewing insects very differently than they do to feeding by phloem-feeding insects (Bidart-Bouzat & Kliebenstein 2011). Plant defense responses to chewing insects are regulated mainly by the JA signaling pathway, with ET playing an additional role (Heidel & Baldwin 2004; Reymond *et al.* 2004; de Vos *et al.* 2005; Ehlting *et al.* 2008). The expression of hundreds of genes changes in response to caterpillar feeding (Reymond *et al.* 2004; Voelckel &

Baldwin 2004; Ehlting *et al.* 2008; Rodriguez-Saona *et al.* 2010; Zhang *et al.* 2013). Genes involved in signaling and secondary chemistry are commonly upregulated, whereas genes involved in photosynthesis and primary metabolism are often downregulated (Voelckel & Baldwin 2004). The transcriptional patterns in response to caterpillar feeding are dynamic over time. For instance, a microarray analysis of *Arabidopsis* in response to feeding of *Plutella xylostella* larvae recorded strong upregulation of wound-response genes involved in octadecanoid biosynthesis over a 24-h period (Ehlting *et al.* 2008). However, SA also seems to be involved in the plant's response to *P. xylostella* feeding, as indicated by upregulation of *PR* genes after 24 h of feeding. Interestingly, *PR* genes are downregulated during early stages of *P. xylostella* feeding (Ehlting *et al.* 2008). Similar responses have been reported in other plant species as well. For example, in tomato, the transcription of *PR* genes was induced by caterpillar feeding (Rodriguez-Saona *et al.* 2010; Kawazu *et al.* 2012). In *N. attenuata*, feeding by various insect herbivores, including the chewing herbivores *Spodoptera exigua, Spodoptera littoralis, Trichoplusia ni* and *Manduca sexta* larvae resulted in increased SA levels (Heidel & Baldwin 2004; Diezel *et al.* 2009). The increased SA levels were consistently correlated with the downregulation of photosynthetic genes (Heidel & Baldwin 2004).

Transcriptomic response patterns in response to multiple attacks

The transcriptomic response to two attackers is far from an additive response to the two attackers individually (Voelckel & Baldwin 2004). For instance, in tomato plants infested by aphids (*Macrosiphum euphorbiae*) and caterpillars (*S. exigua*), the aphids suppressed 27% of the genes regulated by caterpillars, whereas the caterpillars suppressed 66% of the genes regulated by aphids (Rodriguez-Saona *et al.* 2010). In *Arabidopsis*, infestation with the whitefly *Bemisia tabaci* suppresses the upregulation of a large number of genes induced by *P. xylostella* caterpillars (Zhang *et al.* 2013). The interactive effects of two attackers can uncover novel mechanisms. For instance, infestation of *Arabidopsis* plants by *P. rapae* caterpillars induced JA and ET; ET primed the plant for enhanced SA-dependent gene expression in response to infection by turnip crinkle virus (de Vos *et al.* 2006). Transcriptional interference is usually asymmetric. For instance, in *N. attenuata*, transcriptional changes induced by the mirid bug *Tupiocoris notatus* are more resistant to erasure by *M. sexta* caterpillars than vice versa (Voelckel & Baldwin 2004).

Transcriptomic changes occur in distinct patterns and involve large numbers of genes. Analyzing these patterns is usually done with multivariate statistics, but identifying how these transcriptomic changes affect the plant phenotype, especially which genes are responsible for the phenotypic effects and subsequent interactions with members of the insect community, requires a directed approach. In lima bean plants, feeding by *B. tabaci* whiteflies suppressed the induction of the plant's *ocimene synthase* gene, which encodes an enzyme mediating a rate-limiting step

in the biosynthesis of the plant volatile (*E*)-β-ocimene in response to spider-mite feeding. (*E*)-β-Ocimene mediates the attraction of a predatory mite that preys on the spider mite, and whitefly feeding resulted in a reduced attraction of the predatory mite to volatiles from spider-mite-infested plants (Zhang *et al.* 2009).

How to link subcellular mechanisms underlying inducible plant phenotypes to community dynamics

Changes in plant phenotype and their consequences for the plant's interactions with members of the associated insect community take place at very different timescales. Community development takes place on a timescale of weeks to (for perennial woody plants) years, and is based on interactions between individuals that take place on a timescale of days to weeks. These interactions between individuals are affected by changes in the plant phenotype (timescale of hours to days) that are based on transcriptomic changes at a timescale of minutes to days (Figure 3). The different rates at which changes develop at different levels of biological complexity complicate linking these changes causally. For instance, the transcriptome of N. attenuata responds specifically to different herbivore infestations within 24 h, but this difference disappears after 5 days (Voelckel & Baldwin 2004). Linking the transcriptomic response within the first 24 h to community responses at a timescale of weeks to years requires detailed knowledge of how individual species in the community respond to the plastic plant phenotype.

Although understanding how complex molecular changes modulate responses at the community level is a major challenge, detailed knowledge of subcellular mechanisms can provide tools to address this challenge. For instance, knowledge on the involvement of phytohormones can be used to mimic herbivory through the application of a phytohormone. Because JA is one of the major phytohormones involved in plant responses to insect herbivory, it is an interesting initial candidate to manipulate. Pharmacological application of JA to tomato plants has season-long effects on community composition in terms of herbivorous and carnivorous insects. For instance, the abundance of herbivores was reduced and herbivore size was smaller, and these effects on herbivores subsequently affected the performance of predators and parasitoids (Thaler 1999; Thaler *et al.* 2001; Thaler 2002). Applying a single phytohormone at one time point is still a crude method, however, because herbivory results in a dynamic phytohormonal response (Pieterse *et al.* 2012). Pharmacological applications may be made with different phytohormones at different time points (Koornneef *et al.* 2008), but we are not aware of any studies that have investigated the effects of such combinations of applications on community development.

A more accurate approach is to use genetic tools, e.g., by using plants that have been silenced in a single gene involved in the plant's induced response. *N. attenuata*

plants in which a gene encoding for the enzyme lipoxygenase, which mediates the first rate-limiting step in JA biosynthesis, had been silenced were more susceptible to adapted herbivores and attracted novel herbivore species that normally do not feed or reproduce on this plant (Kessler *et al.* 2004). Silencing a gene is quite a drastic manipulation. In nature, plant genotypes more likely differ in relative expression of particular genes, so it will be interesting to monitor community development on different genotypes whose genomes have been (partially) genetically characterized**.** Experiments with genotypes that have not been genetically characterized showed that plant genotypes that differ in secondary metabolites result in considerable variation in community dynamics (Newton *et al.* 2009b; Poelman *et al.* 2009). Community development on different genotypes may converge when the genotypes have been exposed to an early-season specialist herbivore (Poelman *et al.* 2008a; Poelman *et al.* 2010). Because plants in nature are rarely free of herbivory, community dynamics on plants subjected to herbivory are highly relevant to understanding how plant phenotype affects community dynamics.

Data on community development may be linked to transcriptional responses of plants under field conditions (Broekgaarden *et al.* 2010), but this is still far from providing information on the causal links because of the different timescales. Transcriptional responses on a timescale of minutes to days result in a cascade of responses that lead to a dynamic change in plant phenotype. Studying the links between transcriptional dynamics, phenotypic dynamics, and community dynamics requires taking a systems approach that includes experiments in combination with modeling to connect the networks at different levels of biological integration, i.e., the transcriptomic network, the metabolomic network, and the species interaction network (Keurentjes *et al.* 2011).

Sequential changes in herbivore-induced phenotype and community dynamics

Plant–insect interactions represent intricate networks at all levels of biological complexity. These networks consist of hundreds of interacting species at the community level, tens to hundreds of individual insects interacting with a single plant individual, hundreds of plant chemicals that are the product of biosynthetic networks, and hundreds of genes that are regulated by an interacting network of phytohormones. Each of these networks has its own dynamics, and the transcriptomic network that results from herbivore attack affects the biosynthetic network that underlies the change in plant phenotype, which affects interactions with members of the community and consequently community dynamics. Although a systems approach to linking these complex networks at different levels of biological integration will be a major challenge (Keurentjes *et al.* 2011), interesting building blocks are available at the community level with some initial links to knowledge at the mechanistic level**.** Community dynamics result from sequential processes in which the first herbivore's modification of the plant's phenotype then has consequences

for the interactions of the plant with subsequent herbivores (Viswanathan *et al.* 2007; Poelman *et al.* 2008a; Poelman *et al.* 2010; Erb *et al.* 2011). The interaction of a second herbivore with the new plant phenotype may modulate processes at the (sub)cellular level in terms of phytohormonal and transcriptional patterns (Voelckel & Baldwin 2004; Poelman *et al.* 2008a; Rodriguez-Saona *et al.* 2010; Zhang *et al.* 2013), further affecting the plant's phenotype and its interactions with subsequent community members (van Zandt & Agrawal 2004b; van Zandt & Agrawal 2004a; Dicke *et al.* 2009; Zhang *et al.* 2009; Zhang *et al.* 2013). The arrival of these new community members, which now also start to interact with the plant, sets a new round in motion, and so on.

This set of interactions—an herbivore inducing a phenotypic change that then affects subsequent herbivores on the same plant, mediated by induced plant traits—has been termed a trait-mediated interaction unit (TMIU). A TMIU consists of an inducing insect and a plant that mediates the interaction with a second, responding herbivore (Utsumi *et al.* 2010)**.** TMIUs are linked sequentially. This is the case when, for example, a responding herbivore itself becomes an inducer (Utsumi *et al.* 2010), which may happen on both spatial and temporal scales. A spatial chain reaction occurs when the responding herbivore changes its behavior and moves to another plant or plant part (Bukovinszky *et al.* 2010; Utsumi *et al.* 2010) or when responses to feeding herbivores affect herbivores elsewhere on the plant through systemic responses (Utsumi *et al.* 2010; Erb *et al.* 2011). A temporal chain reaction occurs when the responding herbivore later returns to the same plant as an inducer (Underwood 2012) or when the altered plant phenotype affects the performance or population density of the responder, thereby affecting the plant it feeds on (van Zandt & Agrawal 2004b; Utsumi *et al.* 2010).

In fact, several TMIUs might be linked throughout the season, creating a complex indirect interaction web. The resulting cascade shapes the insect community associated with a plant, depending on the first inducing herbivores that arrive on the plant (van Zandt & Agrawal 2004b; Viswanathan *et al.* 2007; Poelman *et al.* 2008a). For instance, on milkweed plants, the identity of the first herbivore early in the season has considerable effects on community development throughout the season (van Zandt & Agrawal 2004b). On *B. oleracea* plants, an early-season, one-week-long infestation by two *P. rapae* caterpillars affected community dynamics throughout the growing season of the plants, with the community on the treated plants comprising more specialist insects than the community on the control plants did (Poelman *et al.* 2008a; Poelman *et al.* 2010). Such cascades may be caused by direct effects of an inducing herbivore on the suitability of the plant to other herbivores and indirect effects of initiating herbivores on the interaction between two or more subsequent herbivores. Herbivores in a TMIU do not all influence the subsequent interactions in the same way, and this may depend on herbivore traits such as feeding guild (Howe & Jander 2008; Bidart-Bouzat & Kliebenstein 2011). Three mechanisms have been proposed by which a plant's physiological response is

directed to (a subset of) certain herbivores: priority effects, overriding effects, and canalization, all of which can be linked to phenomena uncovered at the subcellular level.

Priority effects, overriding effects, and canalization

Priority effects occur when a plant response depends on the order of herbivore arrival on a plant (Miller-Pierce & Preisser 2012)—for example, when the interaction between two herbivores is asymmetrical (Poelman *et al.* 2008a; Erb *et al.* 2011; Miller-Pierce & Preisser 2012; Soler *et al.* 2012a). Asymmetry in these interactions is predominant (Kaplan & Denno 2007), and priority effects are therefore expected to be important in shaping interaction cascades. These asymmetrical priority effects can have several underlying mechanisms, such as competition between the herbivores (Kaplan & Denno 2007; Miller-Pierce & Preisser 2012), which has different outcomes depending on which insect comes first. The kinetics of plant defenses may underlie this. For example, the production of induced plant defense compounds might depend on the sequence of herbivore arrival and can have a larger effect on either the first or the subsequently arriving herbivore (Viswanathan *et al.* 2005; Erb *et al.* 2011). Priority effects may also be mediated by crosstalk between different plant defense pathways, such as the JA-SA crosstalk (Pieterse *et al.* 2012; Thaler *et al.* 2012a).

Overriding effects occur when the inducing effects of one herbivore are overruled by another herbivore on the same plant (van Zandt & Agrawal 2004b; Erb *et al.* 2011). For example, the effects of initial damage to a milkweed plant by monarch caterpillars (*Danaus plexippus*) disappeared when the plant was colonized by other herbivores later in the season (van Zandt & Agrawal 2004b). Moreover, the plant response can also be redirected (Voelckel & Baldwin 2004; Soler *et al.* 2012a) or enhanced (Poelman *et al.* 2008a) following the arrival of subsequent herbivores. Underlying mechanisms may involve irreversible phenotypic changes, such as morphological changes or overriding effects of one signaling pathway on another (Pieterse *et al.* 2012).

Canalization occurs when a first herbivore alone determines the plant's response, regardless of subsequently arriving herbivores (Thaler *et al.* 2002b; Viswanathan *et al.* 2005; Viswanathan *et al.* 2007; Utsumi *et al.* 2010). This effect reduces the plant's ability to be flexible in its response to the herbivore community present at any given point in time, and consequently may affect the development of the herbivore community composition throughout the season. For example, flea beetles affected the number of conspecifics or tortoise beetles throughout the season when arriving first on a plant, irrespective of whether they were followed by tortoise beetles (Viswanathan *et al.* 2007). Underlying mechanisms may include strong and irreversible effects of the phytohormonal signaling in response to the first herbivore or the rapid induction of biosynthetic pathways that result in persistent changes in the plant's phenotype.

Trait-mediated interaction networks and carnivorous insects

The above discussion of trait-mediated interaction networks considered only herbivores in the ecological interactions. However, the third trophic level, consisting of predators and parasitoids of herbivores, also affects the interaction between inducing and responding herbivores (van Veen *et al.* 2006; Utsumi *et al.* 2010). Combinations of multiple herbivores can induce the emission of different blends of plant volatiles (Dicke *et al.* 2009) and thus may attract different predators or parasitoids (Schoonhoven *et al.* 2005). These predators or parasitoids not only decrease the herbivore population by preying on the insects that initially induced the volatiles (Zhang *et al.* 2009; Utsumi *et al.* 2010; Xiao *et al.* 2012) but can also affect other insects, such as herbivores, pollinators, and hyperparasitoids (Dicke & Baldwin 2010; Poelman *et al.* 2012). The events at different moments in time may also be linked, for example, when a predator that is attracted to a plant infested by a first herbivore also preys on other herbivores arriving simultaneously or subsequently on the plant. Different interaction units can occur on a spatial scale as well, when predators induce behavioral changes in herbivores, after which the herbivores move to other plants or plant parts (Utsumi *et al.* 2010).

Because herbivores that are affected by predators and parasitoids can influence the subsequent herbivore community in a cascading manner through priority effects, overruling effects, or canalization, the third trophic level greatly increases the complexity of interactions within a plant–insect community. This is particularly the case when considering multiple initiating herbivores (Zhang *et al.* 2009; Zhang *et al.* 2013).

In summary, interactions between insects associated with a plant are influenced by several factors. The type and sequence of multiple herbivores determine the plant's response, which consequently affects herbivores that subsequently colonize the plant. These secondary herbivores or attracted predators may become inducers in the next plant-mediated interaction unit, which causes a cascade of interactions throughout the insect community.

Future perspectives

The fact that plants are phenotypically plastic in response to herbivore attack contributes to the complexity of plant–insect interactions. For instance, phenotypic plasticity underlies interspecific competition between herbivores at different temporal and spatial scales (Denno *et al.* 1995; Kaplan & Denno 2007). It is important to realize that a plant's genotype determines not only constitutive plant traits but also inducible plant responses, such as the production of metabolites or structural changes. The extent to which constitutive or inducible traits affect plant–insect interactions affects the relative importance of the inducible and the constitutive phenotype for the influence on community dynamics (Poelman *et al.* 2008b; Whitham *et al.* 2012).

In this review, we have focused on the consequences of direct and indirect effects of inducible plant traits on community processes, with a focus on herbivorous and carnivorous insects. Herbivorous insects are connected by both local and systemic plant-mediated interactions. Systemic effects may involve both roots and shoots (Soler *et al.* 2013) or leaves and flowers (Kessler *et al.* 2011; Lucas-Barbosa *et al.* 2011). We have focused on aboveground plant vegetative tissues because most information on community processes is available for insect communities associated with vegetative plant shoots. However, similar systemic effects are expected when including the belowground tissues (Soler *et al.* 2013). Including belowground interactions will be important, even when it further increases the complexity of the interactions and therefore the difficulty of understanding the effects of a phenotypically plastic plant on the development of the associated community. The situation is likely to differ between vegetative and flowering plants because of the major physiological changes that occur during the transition from the vegetative to the reproductive stage. A comparison of vegetative and flowering plants and their associated communities will be interesting to address the different selection forces that these different developmental stages are subjected to.

For the sake of simplicity, we have limited this review to plant–insect interactions. Although insects are the most speciose group within the macrobiome associated with plants (Schoonhoven *et al.* 2005), there is also a speciose microbiome associated with plants (Mendes *et al.* 2011) that represents species with many additional ecological functions, such as pathogens, rhizobia, mycorrhizae, and nonpathogenic rhizobacteria. There is extensive information at the mechanistic, (sub)cellular level for plant–microbe interactions (Pieterse *et al.* 2012), but knowledge of the community processes of microbes associated with plants is much less developed. Nevertheless, it is becoming clear that pathogenic and symbiotic microbes can influence and structure insect communities on plants (Pineda *et al.* 2010; Tack & Dicke 2013). Thus, involving the microbiome in future studies will significantly enhance our understanding of plant–insect interactions. The extensive information on subcellular processes for plant–microbe interactions provides an excellent starting point to manipulate plants via microbes to study the consequences for insect communities. However, including microbial community processes will provide an important new challenge related to the identification of microbes associated with plants (Mendes *et al.* 2011).

Investigating the effects of plants on community development is already a complex task, and unraveling the mechanisms that underlie the community dynamics throughout the season is a significant challenge as well. With a community that, in the case of long-lived plants, can consist of hundreds of species, the number of species combinations involved in plant-mediated interactions seems too large to handle. However, phenological data and natural history data for the system under study may provide a basis for choices that are relevant to the natural situation. For instance, early-season herbivores that predictably occur in the system may have a

prominent effect on plant phenotype that is worth focusing on initially. Furthermore, analyzing community dynamics data through statistical modeling approaches may result in the identification of key species in the community (Keurentjes *et al.* 2011). Such species and the species they interact with may then be the focus of initial studies on underlying mechanisms. Herbivorous insects will be the first group of insects to focus on. When key herbivore species have been selected for such studies, relevant parasitoids and predators should be included next, because their presence and activities affect herbivore behavior (Thaler *et al.* 2012b), population growth (van Veen *et al.* 2005), and interactions with plants (Poelman *et al.* 2011). Again, natural history data may guide the selection of the first species to include in these studies.

The complexity of plant–insect community dynamics and the underlying mechanisms may be overwhelming, and it may seem impossible to understand the processes that shape these speciose and dynamic ecological systems. Rather than stepping back, this complexity should invite directed studies to investigate the ecological processes as well as their underlying mechanisms. Through these studies, we are likely to make small but significant steps toward unraveling how plants influence insect communities. When this has been completed for several different systems, ecological generalities may be identified, and mechanistic knowledge will then allow directed experimental studies to test these generalities. These studies will then enable important progress in understanding interactions between the insects and plants that are so dominant on this planet.

Summary points

1. Plants are members of biodiverse communities consisting of tens to hundreds of species.
2. The insect community associated with plants consists of herbivores at the second trophic level and (hyper)parasitoids and predators at the third, fourth, and higher trophic levels.
3. A plant's phenotypic traits, as determined by its genotype, influence the interactions of the plant with members of the associated community and consequently the community dynamics.
4. A plant's phenotype is highly plastic: Herbivory induces changes in the plant's phenotype, which then influence the plant's interactions with members of the associated community and lead to plant-mediated interactions between community members, such as competition and facilitation.
5. Herbivory induces phytohormonal signaling and transcriptomic rearrangements (timescale of minutes to days) that lead to biosynthetic changes that affect the plant phenotype (timescale of hours to days), with consequences for the plant's

interactions with community members (timescale of days to weeks).

6. Plant responses to herbivores exhibit a considerable degree of specificity. Moreover, the response to two attackers is far from an additive response to the two attackers individually; rather, it involves a strong interaction component that leads to suppression or enhancement of the responses to each herbivore alone, e.g., through phytohormonal crosstalk.
7. The first herbivore-induced change in plant phenotype affects the interactions with subsequently arriving herbivores, which then further affect the phenotype in an interactive way. This sequential process determines community dynamics on a timescale of weeks to years. Thus, the first herbivore that attacks a plant can significantly influence the community dynamics on that plant.
8. Linking herbivore-induced changes in plant phenotype to the ecological consequences that occur at very different timescales is an important multidisciplinary challenge that will provide a comprehensive understanding of how plants interact with their associated communities.

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Chapter 3

Temporal dynamics of plant response to attack by two herbivores on *Brassica oleracea* affects preference and performance of a third herbivore, *Mamestra brassicae*

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Submitted

Abstract

Plants are frequently under attack by multiple insect herbivores, which may interact indirectly through herbivore-induced changes in the plant's phenotype. Various aspects may determine the outcome of these interactions, such as order of and time interval between herbivore arrivals. How variation in induced plant responses caused by different time intervals between two herbivore attackers affects subsequent herbivores has however hardly been investigated.

Here, we tested whether order of arrival and time interval between two inducing herbivores from different feeding guilds affects preference and performance of a subsequently arriving third herbivore, caterpillars of *Mamestra brassicae* L. (Lepidoptera: Noctuidae). Aphids (*Brevicoryne brassicae* L. (Hemiptera: Aphididae)) and caterpillars (*Plutella xylostella* L. (Lepidoptera: Yponomeutidae)) were introduced onto wild *Brassica oleracea* L. (Brassicaceae) plants in different sequences and with different time intervals. The effects of these plant treatments on *M. brassicae* caterpillars were assessed in pair-wise preference tests, and in no-choice performance tests.

The caterpillars of *M. brassicae* preferred to feed on leaf disks from undamaged over double herbivore-induced plants. Compared to leaf disks from undamaged plants, caterpillars preferred to feed on disks of plants that had been first infested by aphids followed by caterpillars, whereas they tended to avoid feeding on leaf disks of plants infested with the reverse herbivore order. Regarding performance, the caterpillars grew better on plants on which two herbivores had been feeding with a longer time interval between them, compared to double herbivory with a short time interval. Although *M. brassicae* grew better on plants induced by aphids than by caterpillars alone, its performance was not affected by the order of arrival of the previous two herbivores.

These results imply that the timing of colonisation by multiple herbivores determines the outcome of plant-mediated herbivore-herbivore interactions. We discuss the consequences of these findings for the dynamics of plant-herbivore communities.

Keywords

Arrival sequence, *Brevicoryne brassicae*, herbivore-induced plant response, performance, *Plutella xylostella*, preference, time interval, wild cabbage

Introduction

At the basis of many aquatic and terrestrial food webs, plants typically are under attack by many herbivore species (Price 2002; Schmitz *et al.* 2004; Schoonhoven *et al.* 2005). Insect herbivores represent the most diverse group of attackers and several species frequently co-occur on individual plants (Schoonhoven *et al.* 2005; Stam *et al.* 2014). Insect herbivores are rarely found to be in strong competition over plant biomass, but instead competition among these herbivores is often mediated indirectly by plant quality (Denno *et al.* 1995; Karban & Baldwin 1997; Kaplan & Denno 2007; Utsumi *et al.* 2010). Herbivores alter plant quality by inducing changes in plant traits such as growth, architecture, resource allocation and mechanical or chemical defence properties (Hunter 1992; Karban & Baldwin 1997; Koricheva *et al.* 2004; Rodriguez-Saona *et al.* 2010). These induced plant phenotypes in turn affect the preference and performance of subsequent herbivores that interact with the herbivore-induced plant, resulting in plant-mediated interactions among herbivores (Utsumi *et al.* 2010; Ohgushi 2016). Because plant responses to herbivores are specific (Agrawal 2000; de Vos *et al.* 2005; Viswanathan *et al.* 2005; Kessler & Halitschke 2007; Bidart-Bouzat & Kliebenstein 2011; Karban 2011), herbivore species identity, order of arrival of multiple herbivores on a plant, and time interval between arrivals are important in shaping indirect plant-mediated interactions (Viswanathan *et al.* 2007; Poelman *et al.* 2008a; Erb *et al.* 2011; Uesugi *et al.* 2013; Stam *et al.* 2016b).

The specificity in plant responses to herbivory creates asymmetry in plant-mediated herbivore interactions (Rodriguez-Saona *et al.* 2005; Kaplan & Denno 2007; Miller-Pierce & Preisser 2012; Ali & Agrawal 2014). Especially species from different feeding guilds induce different plant responses (de Vos *et al.* 2005; Rodriguez-Saona *et al.* 2010; Bidart-Bouzat & Kliebenstein 2011) that may affect subsequent herbivores differentially (Rodriguez-Saona *et al.* 2010; Soler *et al.* 2012a). For example, caterpillars feeding on aphid-induced *Brassica oleracea* plants performed better than on control plants, while aphid performance was not affected on caterpillar-induced plants (Soler *et al.* 2012a). The performance of herbivore species is often negatively affected by plant phenotypes induced by herbivores of the same feeding guild (Rodriguez-Saona *et al.* 2005; Mathur *et al.* 2013).

Most herbivores will encounter a plant that already expresses an herbivore-induced phenotype, often even induced by more than a single herbivore (Karban & Baldwin 1997; Dicke & Hilker 2003; Stam *et al.* 2014). Herbivore food-plant acceptance and performance in these situations, thus, depend on the indirect interaction network between multiple herbivores feeding on the same plant (Utsumi *et al.* 2010). In these interaction networks, the order and timing of herbivore arrival is particularly important as these factors largely determine the plant phenotype expressed after attack by two herbivores, and may thus have unique effects on yet a third herbivore in the interaction network (Viswanathan *et al.* 2007; Utsumi *et al.* 2010; Stam *et al.*

2016b). For example, when a leaf-feeding herbivore had arrived on maize plants before a root-feeding herbivore, the leaf-feeding herbivore negatively affected the performance of the root herbivore. In contrast, when the order of arrival was the reverse, the leaf herbivore did not affect the performance of the root herbivore, suggesting that the induced plant phenotypes differ by the order of herbivore arrival (Erb *et al.* 2011). These effects may be modulated by the time interval between two episodes of herbivore attack, because of temporal aspects of plant physiological responses to herbivory (Kuśnierczyk *et al.* 2008). There may be a time lag between the onset of herbivory and the onset of the plant response (Kuśnierczyk *et al.* 2008; Gomez *et al.* 2010; Karban 2011). When herbivores arrive simultaneously or shortly after each other, they may arrive within the time lag in plant response. This may result in a different plant response compared to when herbivores arrive separated by a long time interval. In that case, the plant may have mounted its responses to the first herbivore and may have to redirect or integrate its induced response to the second herbivore (Karban 2011; Underwood 2012). For example, the longer the time interval between feeding by *Spodoptera exigua* caterpillars on *Plantago lanceolata*, the stronger the negative effect of inducers on the feeding of subsequently arriving conspecifics (Wang *et al.* 2015). Kinetics of the induced plant response can also differ depending on the feeding guild of the inducer (Mathur *et al.* 2013; Mouttet *et al.* 2013; Kroes *et al.* 2016). Moreover, the signal-transduction pathways involved in response to aphid and caterpillar feeding are found to cross-talk and may work antagonistically depending on the order of herbivore arrival (Pieterse *et al.* 2009; Thaler *et al.* 2012a; Wei *et al.* 2014). Therefore, we expect that the time interval between herbivores and order of arrival may particularly interact for plant responses to herbivores of different feeding guilds and thus shape the plant phenotype for subsequent herbivores in the interaction network.

Here, we investigated whether the induced plant phenotype in response to the order of arrival of two herbivores and the time interval between the arrivals affects the preference and performance of a subsequently arriving third herbivore. We conducted two-choice feeding preference tests with the generalist caterpillar *M. brassicae* and examined *M. brassicae* weight gain in performance tests on *Brassica oleracea* plants previously infested by *Brevicoryne brassicae* aphids and/or *Plutella xylostella* caterpillars. All herbivores used in the experiments are known to feed on wild cabbage plants under natural conditions (Moyes *et al.* 2000; Newton *et al.* 2009a). Both *B. brassicae* and *P. xylostella* arrive at similar moments early in the growth season of *B. oleracea* plants (Poelman *et al.* 2009; Poelman *et al.* 2010), which results in variation in arrival pattern of the two herbivores on individual plants. The plants may be colonised first by *B. brassicae* aphids or first by *P. xylostella* caterpillars, with a short to longer time interval between the arrival of the two herbivores on a single plant (Poelman *et al.* 2009; Poelman *et al.* 2010). Subsequently, *M. brassicae* caterpillars that hatch from eggs in these plant-insect communities disperse and search for suitable food plants (Goulson & Cory 1995).

These herbivores are thus exposed to phenotypic variation in *B. oleracea* that is induced by sequential feeding by aphids or caterpillars (Gols *et al.* 2008a; Soler *et al.* 2012a; Li *et al.* 2014; Stam *et al.* 2016b). Our experiments tested the hypothesis that the order of arrival and duration of time interval between two herbivore inducers interact in terms of the effects on the choice for food-plant and performance of a subsequently feeding herbivore. We discuss the implications of our results for plant-mediated interactions among multiple herbivores.

Materials & Methods

Plants and insects

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

Specialist herbivores, i.e., the caterpillars *Plutella xylostella* L. (Lepidoptera: Yponomeutidae) and aphids *Brevicoryne brassicae* L. (Hemiptera: Aphididae), as well as generalist caterpillars *Mamestra brassicae* L. (Lepidoptera: Noctuidae) were reared at the Laboratory of Entomology, Wageningen University, The Netherlands. The insects were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv Cyrus) under greenhouse conditions (21±2 °C, 50-70%RH, 16:8 L:D cycle).

All experiments were conducted under greenhouse conditions (22±5 °C, 50-70%RH, 16:8 L:D cycle), in two subsequent blocks in early- and mid-April 2013.

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

To investigate *M. brassicae* preference for and performance on plants that differed in herbivore-induced phenotype, we induced wild *B. oleracea* plants with either six 2nd larval stage (L2) *P. xylostella* caterpillars and/or fifteen wingless adult *B. brassicae* aphids (Figure 1b). Each species was equally divided over three fully unfolded leaves per plant. During the induction period, plants were individually covered with a fine gauze net to avoid cross-contaminations of insects among plants.

In a full-factorial design, the treatments differed in duration of the time interval and order of arrival of the two herbivores on the plants (Figure 1a). Effects of insect identity on subsequent *M. brassicae* preference and performance were assessed by placing either aphids (A) or caterpillars (C) on the plants, or both simultaneously (B). Furthermore, we placed the two herbivores with a time interval of zero, one or

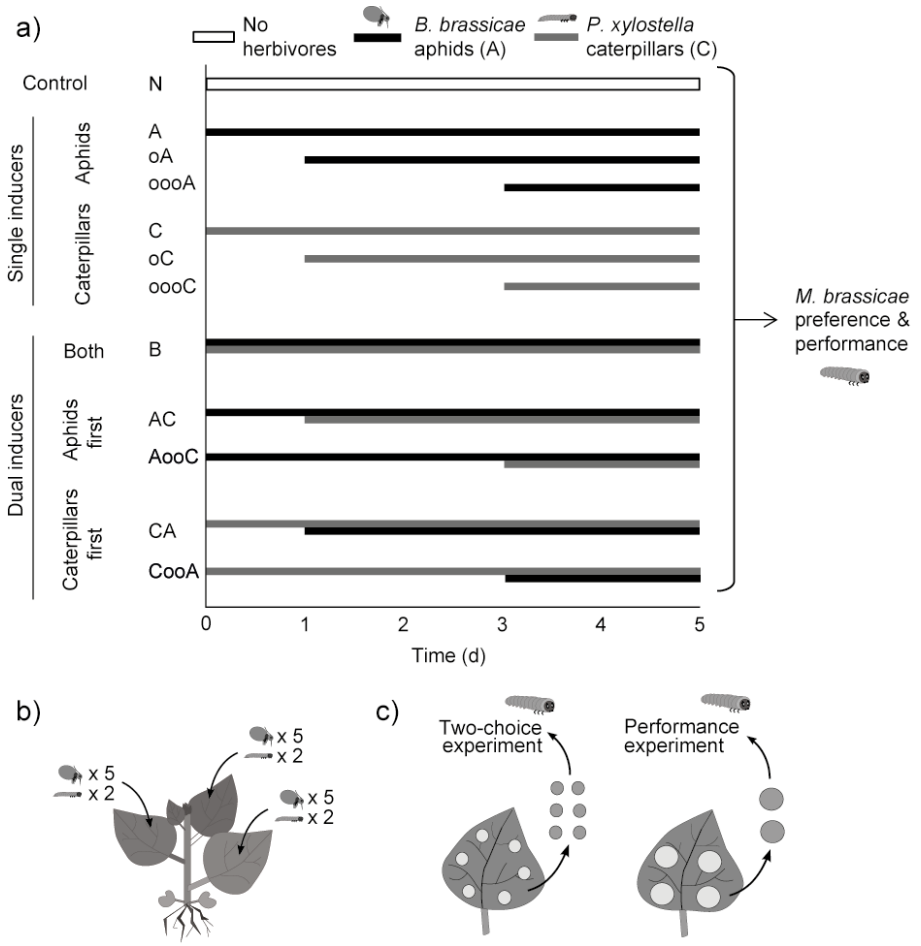


Figure 1. Overview of experimental setup, testing preference and performance of herbivores arriving in different orders and with different time intervals on *B. oleracea*. Figure a) shows the 12 treatments applied to the plants. Treatment capital letters indicate time of induction on the plant, starting from day 0: N: no herbivores; A: aphids *B. brassicae*; C: caterpillars *P. xylostella*; B: both insects simultaneously arriving on day 0; o: no new herbivore induction initiated on that day. Performance of the two inducing herbivores is measured after day 5. *Mamestra brassicae* preference and performance on those induced plants is subsequently tested in a choice test, and after a 48 h-bioassay respectively. Only a subset of plants are used for the *M. brassicae* choice test using only comparisons among treatments that differed most in time interval between inducers (N, A, C, B, CooA, AooC), omitting treatments with 1d interval between the two herbivores and their controls (AC, CA, oC, oA). Figure b) shows the infestation procedure. The three youngest unfolded leaves were infested with five aphids and/or two caterpillars according to the treatments. Figure c) shows the sampling of leaf disks. After the induction period, leaf disks were randomly sampled from each infested leaf. For the two-choice experiment (left), six leaf disks were sampled; for the performance experiment (right), two leaf disks were sampled per induced leaf.

three days on the plants to test whether timing of arrival affects plant resistance to subsequently arriving herbivores. Either aphids or caterpillars were placed first on the plants, resulting in four treatments that differed in time interval and order of arrival (AC, AooC, CA and CooA; with 'oo' indicating two days without insect infestation) and a treatment in which the two herbivores were placed at the same time on the plant (B). Single aphid or single caterpillar treatments placed on plants on the different experimental days were used as control for feeding duration (oC, oooC, oA, and oooA respectively). Undamaged plants with no herbivores (N) were used as overall control. After the last herbivores were placed on the plants, all insects were allowed to continue feeding during two more days, resulting in a total induction period of five days. After this, *M. brassicae* preference and performance tests were conducted (see below).

The presence, order of arrival and time interval between the arrival of inducing herbivores may affect the performance of the inducing herbivores, thereby possibly affecting the strength of induction among treatments. Therefore, the performance of both inducing herbivores was assessed to help explain the preference and performance of the subsequently feeding *M. brassicae* caterpillars. *Brevicoryne brassicae* performance was assessed by counting the total number of aphids (adults + offspring) per plant at the end of the induction period. Each plant was considered a replication unit, with 10-25 replicates per treatment. *Plutella xylostella* performance was assessed by weighing each individual caterpillar at the end of the induction period with a micro balance (Sartorius CP2P, Germany; accuracy 0.001 mg). Individual caterpillars were considered a replication unit, and per treatment 19-88 caterpillars were weighed. Numbers of replicates for both herbivore species varied between treatments due to the use of only a subset of the treatments for the *M. brassicae* preference test and because plants with accidental mechanical damage were eliminated from further experiments and analyses.

Mamestra brassicae caterpillar feeding choice test

In order to test the hypothesis that *M. brassicae* caterpillar feeding preference is affected by the combination of time interval and order of previously arriving herbivores, we used one set of induced plants (described above) to conduct a series of two-choice tests (Figure 1a). We focussed on three sub questions. First, we tested whether *M. brassicae* caterpillars have preference for leaf disks from single aphid (A) or caterpillar (C) induced plants over leaf disks from undamaged plants (N). Second, to test whether time interval and order of arrival between herbivore inducers affects *M. brassicae* food-plant preference, we tested preference for leaf disks of undamaged (N) versus double-herbivore damaged plants and used only the treatments in which the herbivores were introduced in the most widely different time intervals: simultaneous (B), aphids first (AooC) or caterpillars first (CooA). We complemented this by a direct comparison between herbivore order

of arrival (CooA versus AooC). Third, we paired leaf disks from plants induced by single aphids (A), single caterpillars (C), or both herbivores simultaneously (B) with all other treatments (N, A, C, B, CooA, AooC) to gain detailed insight into caterpillar preference for treatments with single- and double-herbivore induction.

Each of the two-choice tests was set up as follows: At the end of the induction period described above, all inducing herbivores were removed from the plants. From each of the three induced leaves per plant, 6 leaf disks were taken (diameter 1.6 cm; total 18 disks per plant), while avoiding visible herbivore damage and main vein (Figure 1c). Leaf disks from different plant individuals within the same treatment were randomly combined with leaf disks from other treatments to ensure samples were independent from each other. In a 5.5 cm Petri dish lined with moist filter paper, two leaf disks from different treatments were placed on opposite sides, and one newly hatched (L1) *M. brassicae* caterpillar was introduced in the middle. Caterpillars were allowed to make a choice by feeding on the two leaf disks for 24h under ambient room temperature conditions. After visual inspection for area of leaf damage, the leaf disk that had most caterpillar feeding damage was considered to be the preferred treatment. When no difference in consumed leaf area between the two disks could be detected or when no feeding occurred, the replicate was scored as 'no-choice'. Each caterpillar was a replication unit, with 51 replicates for each of the choice combinations.

Mamestra brassicae caterpillar performance

To test the hypothesis that *M. brassicae* caterpillar performance is influenced by previous induction by herbivores arriving with different time intervals and in different orders, we set up a feeding-performance experiment. Another set of plants previously induced by *B. brassicae* and/or *P. xylostella* was used to assess *M. brassicae* performance when subsequently feeding on those plants (Figure 1a). All herbivore induction treatments described above were offered to *M. brassicae* caterpillars. To first test the overall effect of the presence and the identity of either or both herbivores on *M. brassicae* caterpillar performance, treatments were categorised as 'no herbivory' (N); insect identity: aphid only (A, oA, oooA), caterpillars only (C, oC, oooC); and both herbivores present (B, AC, CA, AooC, CooA). Effects on the growth of *M. brassicae* caterpillars by the identity of the inducing insect and the time the inducing insect spent on the plant when feeding alone, was subsequently tested using single-herbivore treatments (A, oA, oooA and C, oC, oooC). Finally, to test effects of both the time interval and order of inducing herbivores on *M. brassicae* growth, plants induced with both herbivores were used (B; AC, AooC, and CA, CooA).

At the end of the induction period, all herbivores were removed and from each of the three induced leaves per plant, two leaf disks were taken (diameter 2.4 cm; total 6 disks per plant), avoiding visible herbivore damage and main vein (Figure 1c).

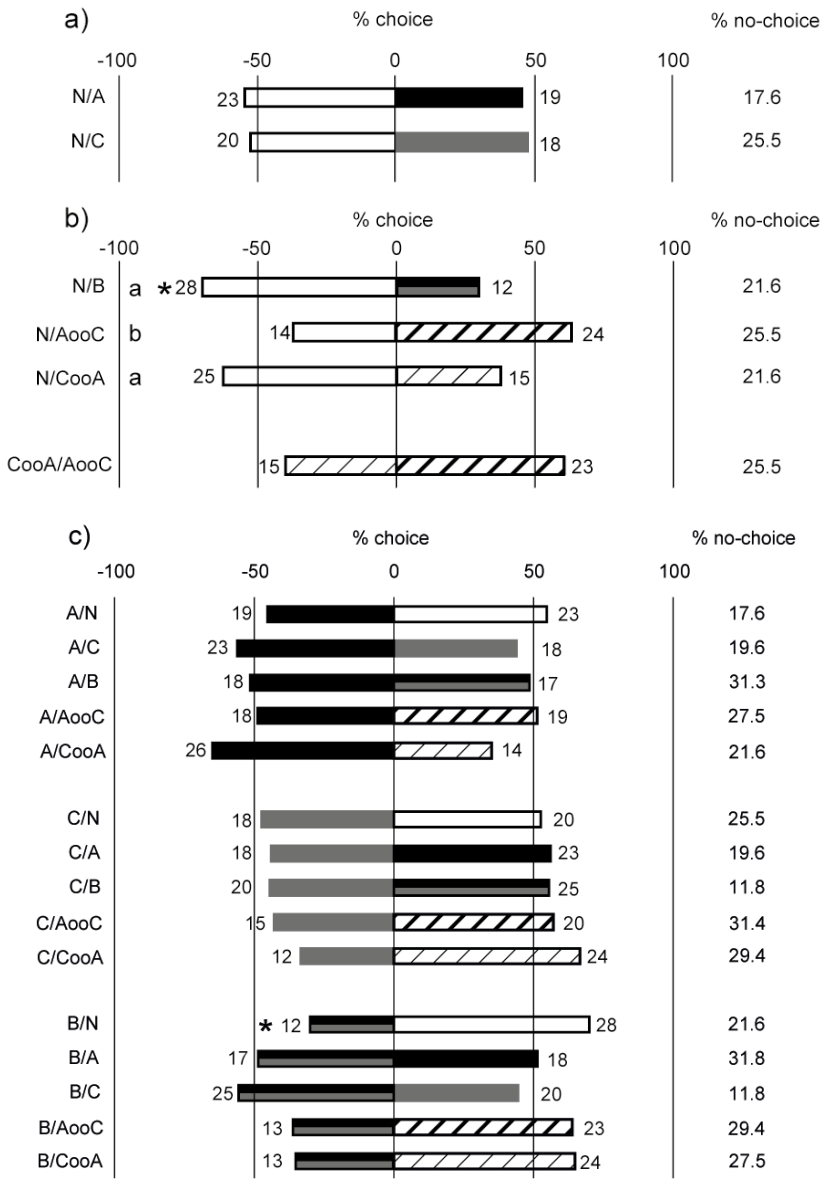
Each leaf disk was individually placed in a 5.5 cm Petri dish lined with moist filter paper. Per dish, one L2-L4 *M. brassicae* caterpillar was carefully introduced, after it had been weighed on an analytical balance (Mettler Toledo ML 4/01, Switzerland; accuracy 0.1mg). Caterpillars were allowed to feed on the leaf disk for 48h under greenhouse conditions. Their feeding and growth was stopped by storing them at 4 °C until weighing again 13 days later with the same balance. Pilot tests demonstrated that storage did not affect caterpillar weight (data not shown). *Mamestra brassicae* absolute growth during the 48h-feeding period was then calculated as [weight before – weight after]. Individual caterpillars were a replication unit, with 39-45 replicates per induction treatment.

Statistical analyses

First, we compared whether the proportion of *M. brassicae* caterpillars choosing for either of the inducing-herbivore treatments differed from a 50:50 ratio using a binomial exact test. Second, we tested differences in choice distributions between different treatment combinations with a Generalized Linear Model (GLM) with binomial distribution and Logit link function. Either herbivore treatment, experimental block, or the interaction between the two were included in the model to obtain Wald statistics for GLM tests for each of the factors and their interaction. Choice distributions were tested among leaf disks of the full-factorial set of induction treatments versus leaf disks of undamaged, aphid-, caterpillar-, or aphid and caterpillar- damaged plants. Choices against leaf disks of undamaged plants were split up in two analyses: undamaged (N) versus either single herbivores (A, C) or dual herbivores (B, AooC, CooA). Finally, differences in the numbers of 'no-choice' (non-responding caterpillars) between treatments, blocks or their interaction were similarly analysed with a GLM.

To meet test assumptions on homogeneity and normality for *M. brassicae* performance, *M. brassicae* caterpillar weight-increase values were square-root transformed prior to analysis. First we tested overall effects of herbivory treatment, experimental block and their interaction on *M. brassicae* performance using a Linear Mixed Model (LMM). Treatment and block were used as main factors and plant identity as random factor. For this overall test, treatments were categorised for no herbivory (N); insect identity: aphid only (A, oA, oooA) or caterpillars only (C, oC, oooC), and both herbivores present (B, AC, CA, AooC, CooA). Then, effects of the identity of single inducing species, the time spent on the plant, the experimental block and their interactions were similarly analysed with an LMM including plant identity as random factor. Finally, we analysed effects of inducing species that arrived first on plants, time interval between inducing herbivores, experimental block and their interactions in similar models, limiting the dataset to treatments in which both inducers were present on the plant.

To compare performance of the inducing herbivores across treatments with different feeding durations, daily increase in aphid population per plant and daily caterpillar growth were calculated by dividing the total number of aphids or caterpillar weight by



the number of days they spent feeding on the plant. Daily increase in aphid numbers was ^{10}log transformed to meet test assumptions. The effects of herbivory treatment, block, and their interaction were tested with two-way ANOVA, followed by LSD post-hoc tests if results were significant. To meet test assumptions, caterpillar weights were double log transformed (x'=log[-log(x/100)]) prior to analysis on effects of herbivore treatment, block and their interaction with an LMM. Treatment and block were used as main factors and

◄ **Figure 2.** *Mamestra brassicae* caterpillar preference for plants previously induced by different order and timing of arrival of two herbivores. Bars show the percentage of *M. brassicae* choices for either of two treatments in a two-choice test and within each bar the absolute numbers of choosing *M. brassicae* caterpillars. Treatment capital letters indicate time of induction on the plant with aphids *B. brassicae* and/or caterpillars *P. xylostella*, starting from day 0: N: no previous herbivory; A: aphids, C: caterpillars; B: both insects simultaneously arriving on day 0; o: no new herbivore induction initiated on that day. Figure a) shows choice tests of single inducing herbivores compared to undamaged plants; Figure b) shows choice tests of dual inducing herbivores compared to undamaged plants; while c) shows choice tests compared to either aphid-, caterpillar- or simultaneously-induced plants. Some choice-combinations are shown repeatedly for clarity of comparisons. In Figure b), choice-combination 'CooA/AooC' was not included in GLM test for different choice distributions among choice-combinations (versus undamaged plants). Asterisk (*) indicate choices that significantly differed from 50:50 choice ratio, while different lower case letters indicate choice-combinations that significantly differ among each other. Column on the right indicates percentage of non-responsive *M. brassicae*.

Table 1. Generalized Linear Model Wald table for *M. brassicae* caterpillar choice distributions and unresponsiveness. Differences in *M. brassicae* choice distributions and no-choice distributions (not responding caterpillars) between treatments, blocks, or their interaction. Treatments were two-treatment combinations in a full factorial design of herbivore-induced plants against undamaged plants (N), aphid-damaged (A), caterpillar-damaged (C) or plants damaged by both herbivores (B). GLM tests against undamaged plants were split up into 'single herbivores against N' (N/A, N/C) and 'dual herbivores against N' (N/B, N/AooC, N/CooA). Wald indicates Wald statistic, and P gives Chi-square probability. Numbers in **bold** indicate significant effects (α=0.05).

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

plant identity as random factor, followed by an LSD post-hoc test if results were significant. LMMs on *M. brassicae* and *P. xylostella* performance, and binomial exact test and GLM on *M. brassicae* choice and no-choice were conducted with GenStat software Version 17.1 (VSN International, Hemel Hempstead, UK). An ANOVA was conducted with SPSS Version 22.0.0.1 to analyse *B. brassicae* performance (Armonk, NY, USA: IBM Corp.).

Results

Mamestra brassicae caterpillar preference

The number of non-responding *M. brassicae* caterpillars in the pair-wise choice tests (11-32%, Figure 2) was not affected by the different choice-combinations that were offered to the caterpillars (Table 1). The choice of *M. brassicae* caterpillars for leaf disks was not strongly affected by previous herbivory on those leaves, since most of the preference tests did not yield significant results. However, *M. brassicae* caterpillars preferred to feed on leaf disks of undamaged plants over leaf disks of plants infested simultaneously by aphids and caterpillars (N vs B; Figure 2). In all other pair-wise choices between leaf disks of undamaged versus herbivore-induced plants, and aphid-, caterpillar- and double- induced versus all other treatments in the full-factorial design, *M. brassicae* did not show a feeding preference for one of the treatments deviating from a 50:50 ratio (Figure 2).

However, the distribution of *M. brassicae* choices among the herbivore-treatment combinations that were offered revealed some differences (Table 1). Although *M. brassicae* did not choose differently for either aphids alone or caterpillars alone over undamaged leaf disks (Figure 2a), choices for undamaged leaf disks versus leaf disks damaged by both herbivores differed significantly depending on the order of arrival and time interval between the arrival of those herbivores (Table 1; Figure 2b). *Mamestra brassicae* consumed more frequently a larger leaf area from disks of plants induced by aphids followed by caterpillars (AooC) than from leaf disks of undamaged plants in paired choice tests, whereas leaf disks of plants induced by caterpillars and, subsequently, by aphids (CooA), or of plants that were induced by both herbivores simultaneously (B), were avoided over leaf disks of undamaged plants (lower case letters in Figure 2b; LSD comparisons: N/AooC vs N/CooA: P=0.025; N/AooC vs N/B: P=0.004; while N/B vs N/CooA: P=0.478). In line with this observation, when comparing only between the different orders of herbivore arrival (caterpillars first (CooA) or aphids first (AooC)), the direction of *M. brassicae* preference was similar as in the comparisons against undamaged leaves. *Mamestra brassicae* caterpillars fed more frequently a larger area from leaf disks of plants that were first induced by aphids followed by caterpillars (AooC) than from leaf disks induced in the reverse order (CooA), although this comparison was not significantly different from a 50:50 ratio (Figure 2b).

In choice tests between leaf disks of plants induced by single herbivores (aphid (A) or caterpillar (C)) or both herbivores simultaneously (B) *versus* all other treatments in the factorial design, the distribution of *M. brassicae* choices did not vary significantly between any of the two-choice combinations (Figure 2c; Table 1).

Table 2. General Linear (Mixed) Model for herbivore performance. Differences in overall *M. brassicae* performance after feeding on plants induced by different herbivory treatments, blocks, and their interaction. Difference in performance of the inducers *B. brassicae* and *P. xylostella* after induction period on plants induced by herbivory treatments, blocks, and their interaction. *Mamestra brassicae* and *P. xylostella*: Linear Mixed Model, with plant identity as random factor included in the model; *B. brassicae*: ANOVA. *Mamestra brassicae* performance was tested for overall effects of herbivory treatments: no herbivory (N); insect identity: aphid only (A, oA, oooA), caterpillars only (C, oC, oooC); and both herbivores present (B, AC, CA, AooC, CooA). Numbers in **bold** indicate significant effects (α=0.05).

	Treatment			Block			Treatment * Block		
	df	F	P	df	F	P	df	F	P
M. brassicae performance; overall	3	1.72	0.166	1	143.37	**<0.001**	3	0.40	0.754
B. brassicae performance	7	3.324	**0.003**	1	118.062	**<0.001**	7	0.846	0.552
P. xylostella performance	7	18.20	**<0.001**	1	73.24	**<0.001**	7	2.92	**0.007**

Table 3. General Linear Mixed Model for *M. brassicae* performance on single- or dual-species induced plants. Difference in *M. brassicae* performance after feeding on plants induced by different herbivory treatments: Linear Mixed Model, with plant identity as random factor included in the model. Factors tested as followed: When *M. brassicae* was feeding on plants with single herbivores only (A, oA, oooA, C, oC, oooC), effects of Time herbivores spend on the plant (5d, 4d or 2d), Species (aphids or caterpillars), Blocks, and all interactions were tested. When *M. brassicae* was feeding on plants with dual herbivory only (B, AC, AooC, CA, CooA), effects of Time interval between herbivore arrival (0d, 1d or 3d), Species order of arrival (aphids first or caterpillars first), Blocks, and all interactions were tested. Numbers in **bold** indicate significant effects (α=0.05).

	Time (1)			Species (2)			Block (3)		
	df	F	P	df	F	P	df	F	P
M. brassicae performance; single herbivory	2	0.56	0.573	1	4.49	**0.035**	1	91.43	**<0.001**
M. brassicae performance; dual herbivory	2	3.93	**0.028**	1	2.46	0.125	1	37.54	**<0.001**

	1 x 2			1 x 3			2 x 3			1 x 2 x 3		
	df	F	P	df	F	P	df	F	P	df	F	P
M. brassicae performance; single herbivory	2	0.12	0.890	2	1.41	0.247	1	0.04	0.839	2	1.64	0.196
M. brassicae performance; dual herbivory	1	0.48	0.491	2	0.75	0.482	1	0.86	0.359	1	1.75	0.195

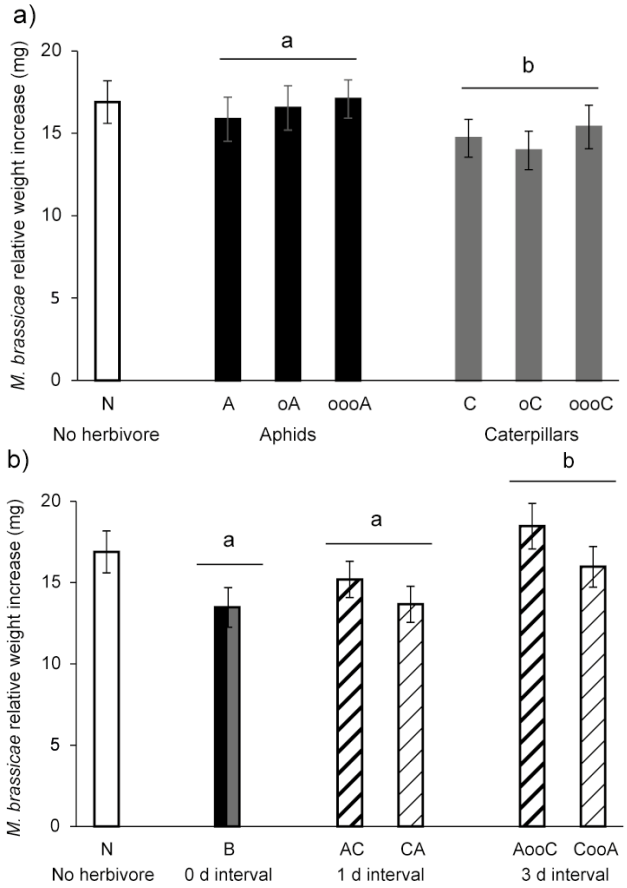


Figure 3. *Mamestra brassicae* caterpillar performance when feeding on plants previously induced by different order and timing of arrival of two herbivores. Average weight increase (weight after - weight before), ± SE. Treatment capital letters indicate time of induction on the plant with aphids *B. brassicae* and/or caterpillars *P. xylostella*, starting from day 0: N: no previous herbivory; A: aphids, C: caterpillars; B: both insects simultaneously arriving on day 0; o: no new herbivore induction initiated on that day. For comparison, treatment 'N' is included in the graphs, although this treatment is only included in overall analysis. Figure a) shows *M. brassicae* performance on plants previously induced with single herbivores, while figure b) shows *M. brassicae* performance on plants previously induced with both herbivores. Different lower case letters indicate groups that significantly differ.

<u>*Mamestra brassicae* caterpillar performance</u>

When comparing in an overall comparison the performance of *M. brassicae* caterpillars in terms of weight gain on plants with either no previous inducers, only aphids, only caterpillars or both inducers, no significant differences were recorded (Table 2). However, when comparing only plants with single inducers, *M. brassicae* caterpillar performance was affected by the species of inducer (Table 3). *Mamestra*

brassicae had a 12% higher weight increase when feeding on aphid-induced plants than when they were feeding on caterpillar-induced plants (Figure 3a). For both caterpillar and aphid infestations, the duration of prior induction of 2, 4 or 5 days did not significantly affect the performance of subsequently arriving *M. brassicae* caterpillars (Table 3).

However, the timing of induction was important for the performance of *M. brassicae* when feeding on leaf disks of plants induced by both caterpillars and aphids (Table 3). Pair-wise comparisons show that *M. brassicae* gained more weight on plants exposed to dual infestation when the time interval between infestation with aphids and caterpillars was long (3 days) than when the time between infestations was short (1 or 0 days; Figure 3b). *Mamestra brassicae* grew on average 28% more on plants with a 3-day interval between inducing-herbivores compared to a 0-day interval (LMM: $Wald_1$:6.84, P=0.009), and 19% more compared to a 1-day interval between the inducers (LMM: $Wald_1$:4.28, P=0.047); while the 7% increased growth on plants with 1 day between inducers was not significantly different from performance on plants with simultaneous infestation of both herbivores (LMM: $Wald_1$:0.69, P=0.406). The order of herbivore infestation on the plants (aphids first or caterpillars first) did not affect the performance of *M. brassicae* (Table 3). Also no interaction was found between order of arrival and the time lag in arrival of the two herbivores on the growth of subsequent feeding *M. brassicae* caterpillars (Table 3).

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

During the induction period, the performance of *B. brassicae* aphids and *P. xylostella* caterpillars was affected by the feeding duration and the presence of the other inducer, revealing an interaction between their order of arrival and time interval (Table 2).

When alone on the plant, the daily increase in *B. brassicae* population was reduced with a longer time spent on plants by the aphids (Supplementary Figure 1). When together with *P. xylostella* on the plant, the aphid population increase was positively affected by a longer time interval between the two herbivores, but only when caterpillars had arrived first on the plants (Supplementary Figure 1).

When caterpillars were feeding alone on the plant, the daily weight gain of *P. xylostella* increased the longer they spent feeding (Supplementary Figure 2). When together with *B. brassicae* on the plant, the order of arrival of the two herbivores reversed the effect of time interval between infestations with the two herbivores. With caterpillars arriving first, caterpillar growth increased with a longer time interval between herbivores, while with aphids arriving first, caterpillar growth decreased with a longer time interval between herbivore infestations (Supplementary Figure 2).

Discussion

Plant-mediated interactions between herbivores have a dynamic character in nature. The consequences of the dynamics between two herbivores for other community members due to variation in the timing of herbivore colonisation on an individual plant are still poorly understood (Gomez *et al.* 2010; Karban 2011; Underwood 2012). We found that preference and performance of *M. brassicae* caterpillars were affected when they were feeding on cabbage plants whose phenotype was altered by previous herbivores that arrived in different orders and with different time intervals.

The order of herbivore infestation affected *M. brassicae* feeding preference. *Mamestra brassicae* caterpillars fed more frequently on leaf disks from plants on which aphids had arrived before caterpillars over leaf disks from undamaged plants. However, leaf disks from plants on which caterpillars had arrived first or had arrived simultaneously with aphids received less damage than leaf disks from undamaged plants. The order of aphid and caterpillar infestation had no effect on the growth performance of *M. brassicae*. However, *M. brassicae* caterpillars grew better on plants previously induced with only aphids than on plants induced with only caterpillars.

The time interval between arriving herbivores did not strongly influence the preference of *M. brassicae* caterpillars. However, *M. brassicae* weight increase was larger, the longer the time interval was between the two inducers. This was not due to the difference in the time the inducers spent feeding on the plant, as *M. brassicae* did not experience a growth difference between plants with various feeding durations of aphids alone or caterpillars alone. The feeding preference and performance of *M. brassicae* were not affected by the interaction of the order and time interval between previous feeding by two herbivores.

Finally, the inducers themselves were affected by the presence and timing of arrival of the other herbivore, but this did not directly explain feeding preference or performance of *M. brassicae*. *Brevicoryne brassicae* and *P. xylostella* performance were affected by the feeding duration when feeding alone, as well as the interaction between timing and order of arrival.

Often, lepidopteran preference tests are carried out with an oviposition test of the adult female, and performance tests with feeding offspring. However, sometimes a discrepancy between the two exists due to different selection pressures on the two life stages (Wiklund 1975; Schoonhoven *et al.* 2005; Gripenberg *et al.* 2010; Soler *et al.* 2012b; Gómez Jiménez *et al.* 2014). Instead of adult moth oviposition choice, we allowed neonate caterpillars to choose between differently induced plants. These caterpillars are known to search for their own food source after hatching (Goulson & Cory 1995). After the choice for a suitable food source has been made, the performance of the herbivore on that plant indicates the nutritional and toxic value

of the herbivore-induced plant phenotype (Karban & Baldwin 1997; Hopkins *et al.* 2009). Also for caterpillars, the preference for and performance on a food source do not always match (Wiklund 1975; Schoonhoven *et al.* 2005). Here, we mostly observed either similar patterns in choices and growth increase of *M. brassicae* caterpillars, or no strong effect on caterpillar choice. This indicates that *M. brassicae* caterpillars can select a food source suitable for further development or at least do not make unfavourable choices (van Leur *et al.* 2008; Harvey & Gols 2011a).

Identity and order of arrival of inducing herbivores

Mamestra brassicae caterpillars grew faster on aphid-induced plants than on caterpillar-induced plants. This pattern fits in the pattern of differential induction of phytohormonal pathways by herbivores from different feeding guilds (Thaler *et al.* 2002b; Voelckel & Baldwin 2004; de Vos *et al.* 2005; Ali & Agrawal 2014). The phytohormonal pathway generally induced by phloem feeders such as aphids negatively interferes with the phytohormonal pathway induced by caterpillars, thus alleviating plant resistance against caterpillars (Zarate *et al.* 2007; Soler *et al.* 2012a; Zhang *et al.* 2013).

Here, not only the identities of the inducing herbivore species were important for subsequent feeding choice of *M. brassicae*, but also the order in which they infested the plant. The importance of herbivore order of arrival has been previously identified in pair-wise interactions among above- and belowground herbivores (Erb *et al.* 2011; Wang *et al.* 2014). These studies identified that the first-arriving herbivore had negative effects on the second entrant, but the second herbivore had neutral effects on the performance of the first herbivore (Erb *et al.* 2011; Wang *et al.* 2014). Beyond these pair-wise interactions, the order of herbivore arrival has also been found to affect subsequent community members later in the season (Viswanathan *et al.* 2007; Miller-Pierce & Preisser 2012; Stam *et al.* 2016b). For example on *Solanum dulcamara* plants, the first-arriving insect always determined the numbers of herbivores on that plant later in the season (Viswanathan *et al.* 2007). This could indicate that the plant prioritizes its responses to the first-arriving herbivore (Viswanathan *et al.* 2007; Miller-Pierce & Preisser 2012; Stam *et al.* 2014). However, studies focussing on plant responses by measuring transcriptome responses to double-stress suggest that the second inducer at least leaves a strong signature on plant responses shortly after feeding (Voelckel & Baldwin 2004; Coolen *et al.* 2016; Davila Olivas *et al.* 2016). It remains to be identified whether these translate into strong phenotypic changes or that this discrepancy in literature is caused by specificity of double-stress in different plant species. Our data are in line with the pattern in literature on herbivore responses to induced plants (Thaler *et al.* 2002b; Zhang *et al.* 2009; Soler *et al.* 2012a; Mathur *et al.* 2013): *M. brassicae* preferred plants on which aphids had arrived first better than undamaged plants (AooC/N), but it avoided plants on which caterpillars had arrived first compared to

undamaged plants (CooA/N), regardless of simultaneous or subsequent induction by the other herbivore. Because *M. brassicae* grew better on aphid-induced than on caterpillar-induced plants, this indeed hints towards a stronger plant resistance response to the first inducer than towards the second inducer. Moreover, our data show that these processes may be influenced by the time interval between arrival of different herbivores.

Time interval between inducing herbivores

Mamestra brassicae caterpillars performed better on plants that were induced by double herbivore attack with a longer time interval between infestation by the two herbivores. This contrasts to a study on *Plantago lanceolata* in which a longer time interval between the arrival of conspecifics caused a decrease in the consumption of leaf area by *Spodoptera exigua* caterpillar (Wang *et al.* 2015). However, in another study with *S. exigua* caterpillars attacking tomato plants twice with a short or longer period between the two attacks, a short time interval yielded a lower plant resistance response compared to the plant response after the first attack only (Underwood 2012). In contrast, no decrease in plant resistance was observed with a longer time interval between infestations by the herbivores (Underwood 2012). This indicates that a response to a first herbivore attack limits plants in the strength and speed of a response to a second attacker (Karban 2011; Underwood 2012).

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

of caterpillars on a plant phenotype induced by two herbivores with a long time interval between them, as was found for *M. brassicae* in our study.

As plant responses to herbivores from different feeding guilds are often asymmetric (Kaplan & Denno 2007), and we observed that the time interval between herbivore arrivals affects the performance of a subsequent herbivore (Underwood 2012; Wang *et al.* 2015), we expected an interaction between the effects of both order of inducer arrival and time interval on *M. brassicae* performance. However, we found no evidence for such an interaction effect here. This is in contrast to a study with powdery mildew and whiteflies on tomato plants that showed reverse effects of time interval between the attackers when their order of arrival changed (Mouttet *et al.* 2013). Apparently, in our case the time interval between herbivores shapes the plant phenotype such that it affects the performance of a subsequent feeder, but the order of herbivore species arrival did not matter. The mechanisms that underlie these effects remain to be elucidated.

Performance of inducers

Most studies on plant-mediated herbivore-herbivore interactions focus on two species, but do not investigate how their responses might affect plant phenotypic consequences for subsequent feeders (Utsumi *et al.* 2010; Stam *et al.* 2014; Utsumi 2015). Our study shows that performance of the two inducing herbivores indeed was also affected by the presence of the other herbivore on the same plant for varying time durations. However, not all these aspects were reflected in the performance of subsequent feeding by *M. brassicae*. This indicates that plant responses that mediate indirect interactions between multiple herbivores do not directly translate with the same magnitude and direction into responses of another herbivore (Utsumi *et al.* 2010; Ohgushi 2016). The response of an herbivore to an induced-plant phenotype depends on many aspects of plant-herbivore interactions, such as the species and feeding guild of inducing and responding herbivore (Bidart-Bouzat & Kliebenstein 2011; Ali & Agrawal 2014), the type of plant responses involved (Howe & Jander 2008; Rodriguez-Saona *et al.* 2010), and modifications of the plant response due to timing and order of arrival of multiple herbivores (Erb *et al.* 2011; Karban 2011; Stam *et al.* 2014; Wang *et al.* 2015). Therefore, going from two to multiple herbivores in more natural situations of plant-herbivore communities cannot be interpreted by simple extrapolations (Stam *et al.* 2014; Poelman 2015).

Conclusion and future perspectives

Here, we show that the timing of arrival of two herbivores on a plant changes the plant phenotype such that it affected a subsequent feeder in its choice and performance. The outcome of plant-mediated interactions among multiple herbivores in a community may thus be subject to variation in order of arrival and

time interval among herbivores. Major challenges in plant-insect interactions are to understand the mechanisms that shape these interaction networks and how these networks are reflected in evolutionary processes of plant-insect interactions (Utsumi *et al.* 2010; Poelman 2015; Ohgushi 2016; Poelman & Kessler 2016).

To understand how plant responses to herbivory shape interaction networks, more knowledge is needed about the kinetics of plant responses to herbivory and how these physiological processes influence plant responses to multiple herbivore attack. Especially the time lag before onset of plant responses, changes in response strength during herbivore feeding and the decay of plant response after feeding has stopped are poorly understood (Gomez *et al.* 2010; Karban 2011; Underwood 2012). A next step to understand nature's complexity would then be to unravel the kinetics of plant responses to herbivores from different feeding guilds, which induce different types of plant response (Bidart-Bouzat & Kliebenstein 2011; Erb *et al.* 2011; Karban 2011). Each of these aspects may then determine how a plant responds to multiple herbivore attack by integrating physiological responses to multiple attackers.

Second, how these plant phenotypes, shaped by induction of multiple herbivores, determine food-plant preference of other community members is key in understanding how insect communities on individual plants are structured. It requires identification whether herbivores select for plant phenotypes induced by multiple herbivores or that their food plant preference is determined by presence of a specific key herbivore (Utsumi *et al.* 2010; Ohgushi & Hambäck 2015; Poelman & Kessler 2016). The latter was identified for parasitic wasps in their search for hosts that were accompanied by multiple herbivore species on a single plant (de Rijk *et al.* 2016).

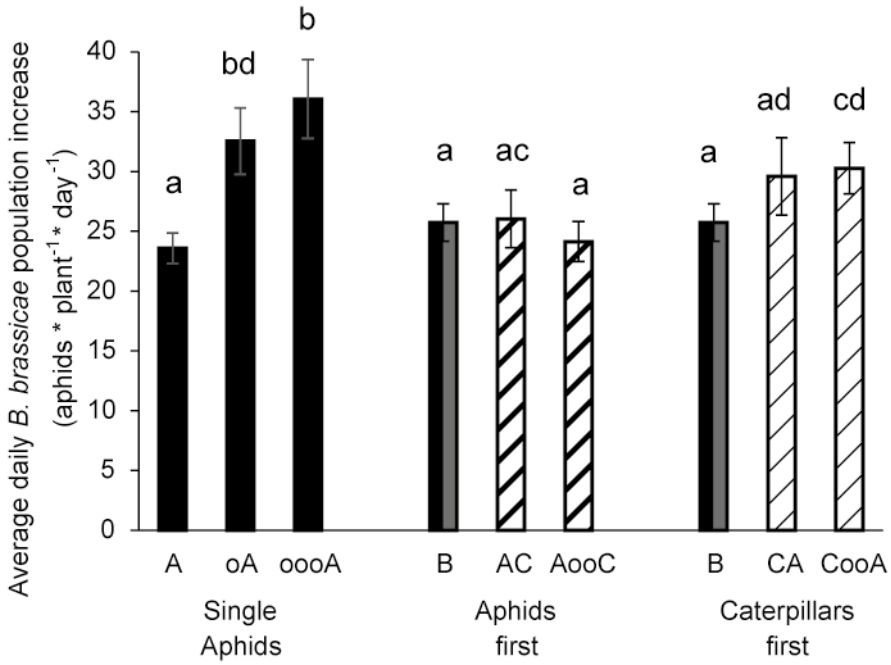
Finally, the feedback loops of herbivory to plant phenotype and back to herbivores exert evolutionary selection pressures on all parties (Utsumi *et al.* 2013; Ohgushi 2016). Plants are expected to respond to such dynamic, continuously changing interaction webs, but whether and how this occurs is still largely unknown (Stam *et al.* 2014; Wurst & Ohgushi 2015; Poelman & Kessler 2016).

Acknowledgements

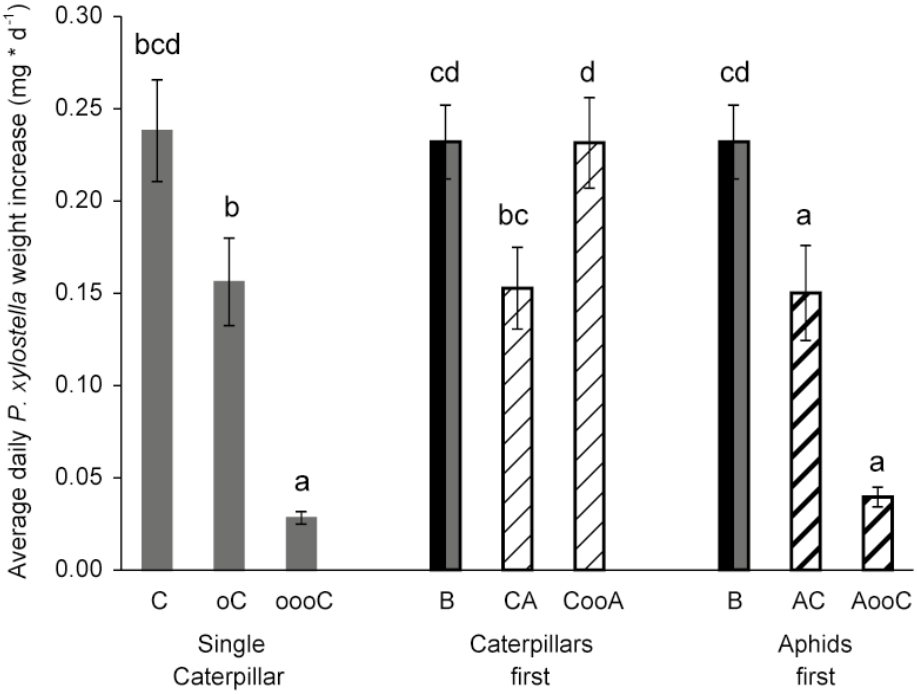
We thank Léon Westerd, André Gidding and Joop Woelke for rearing the insects and Unifarm for maintaining the greenhouses. The *B. oleracea* seeds were kindly provided by Rieta Gols. This study was supported by a TOP GO grant (no. 854.10.010 to Marcel Dicke) from The Netherlands Organization for Scientific Research (NWO), and by a personal grant to Lucille Chrétien, funded by the French Ministry of Research and Higher Education and delivered by the École Normale Supérieure de Lyon.

Chapter 3

Supplementary material



Supplementary Figure 1. *Brevicoryne brassicae* aphid performance when feeding during different periods before or after or together with *P. xylostella* caterpillars. Daily aphid population increase per plant, ± SE. Treatment capital letters indicate time of induction on the plant, starting from day 0: B: both insects arriving simultaneously on day 0; A: aphids; C: caterpillars; o: no new herbivore induction initiated on that day. Treatment 'B' is shown twice for clarity of comparisons. Different lower case letters indicate significant different groups.



Supplementary Figure 2. *Plutella xylostella* caterpillar performance when feeding during different periods before or after or together with *B. brassicae* aphids. Average increase of caterpillar weight per day, un-transformed data, ± SE. Treatment capital letters indicate time of induction on the plant, starting from day 0: B: both insects arriving simultaneously on day 0; C: caterpillars; A: aphids; o: no new herbivore induction initiated on that day. Treatment 'B' is shown twice for clarity of comparisons. Different lower case letters indicate significantly different groups. Statistical analysis of these data was conducted on double-log transformed data to meet test assumptions, hence the post-hoc lower-case letters should not be extrapolated based on the current figure presenting the untransformed data.

Chapter 4

Order of herbivore arrival on wild cabbage populations influences subsequent arthropod community development

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Submitted

Abstract

Density- and trait-mediated processes shape community dynamics in various ecosystems. Insect community dynamics are shaped by indirect plant trait-mediated interactions, which cascade through the community in a spatial-temporal manner. Both the specificity of plant responses to single herbivores as well as unique responses to sequential or simultaneous attack by multiple herbivores may determine the plant trait-mediated effects on subsequent herbivores.

How order of insect species arrival affects community development is not well understood. Under field conditions we investigated the effects of colonization order by early-season herbivore species of different feeding guilds on community dynamics during two growth seasons. Furthermore, specificity of the order of herbivore arrival was studied for two wild *Brassica oleracea* plant populations.

Both identity of the early herbivores and the order of their arrival affected the later arthropod community development in a non-additive manner. Moreover, the effects were specific for the plant population on which the interactions occurred. Herbivore density-dependent effects as well as induced plant trait-mediated interaction cascades played a role in shaping the community. This indicates that sequences of herbivore colonization on a plant early in the season have long-term consequences for later feeding pressure. We further discuss how kinetics of the underlying induced plant responses may influence community dynamics and ultimately plant fitness.

Our work shows that community processes, such as indirect plant-mediated species interactions may have important implications for trait evolution in individual community members.

Keywords

Arrival sequence, *Brassica oleracea*, community dynamics, herbivory, induced response, plant mediated insect interactions, plant population

Introduction

Trait- and density-mediated processes may profoundly shape community dynamics in terrestrial and aquatic ecosystems (Trussell *et al.* 2003; Křivan & Schmitz 2004; Schmitz *et al.* 2004; van Veen *et al.* 2006). Trait-mediated interactions in which one organism, through its traits, affects another through an intermediate species cause indirect linkage between organisms sharing the same resource (Werner & Peacor 2003). This results in horizontal species interactions as well as in connecting species from widely different functional groups that have no trophic relationship, such as elephants and lizards, herbivores and pollinators or bivalves and barnacles (Paine 1966; Ohgushi 2005; Kessler & Halitschke 2007; Pringle 2008). In addition to trait-mediated effects, density-mediate effects may also play a role (Utsumi *et al.* 2010) and both may be connected. For instance, herbivore density may influence the strength of trait-mediated effects (Zhang *et al.* 2009; Utsumi *et al.* 2010; Ando *et al.* 2011; de Rijk *et al.* 2013; Kroes *et al.* 2015). These density- and plant-mediated herbivore interactions may cascade into effects on higher trophic levels separated in space and/or time and *vice versa* higher trophic levels may modulate these interactions by consumptive and non-consumptive effects on herbivores (van Zandt & Agrawal 2004b; Poelman *et al.* 2008a; Kaplan & Thaler 2010; Utsumi *et al.* 2010; Erb *et al.* 2011; Finke 2012; Stam *et al.* 2014; Kroes *et al.* 2016).

In insect-plant interactions, indirect trait-mediated interactions are particularly important for community dynamics on individual plants and result in intricate indirect interaction networks (Utsumi *et al.* 2010). In these networks, plant responses to a first herbivore affect the likelihood of plant colonization by and performance of a subsequent herbivore and these second colonizers in turn modulate the plant phenotype by their own feeding-induced plant responses. Because plant physiological responses to single and multiple herbivores are highly specific (Soler *et al.* 2012a; Ali & Agrawal 2014), the order and time lag between herbivore arrival may thereby differentially affect insect community development (Viswanathan *et al.* 2007; Erb *et al.* 2011; Miller-Pierce & Preisser 2012). Combinations of early-season herbivores can result in various induced plant phenotypes due to differences in herbivore feeding mode, plant species or genotypic variation in induced responses to herbivory (Agrawal 2000; Erb *et al.* 2012; Agrawal *et al.* 2014). Herbivores from different feeding guilds are well known to induce different types of phenotypic changes in plants by triggering different plant hormone-associated pathways, such as the jasmonic acid (JA) and ethylene (ET) pathways by chewing caterpillars and the salicylic acid (SA) pathway by phloem-feeding aphids (de Vos *et al.* 2005; Diezel *et al.* 2009; Bidart-Bouzat & Kliebenstein 2011; Vos *et al.* 2013a). Moreover, cross-talk between the pathways can occur upon multiple herbivory (either simultaneous or subsequent) (Voelckel & Baldwin 2004; Rodriguez-Saona *et al.* 2010; Thaler *et al.* 2012a), thereby causing specific plant responses upon different combinations

of multiple herbivory (Soler *et al.* 2012a; Mathur *et al.* 2013; Ali & Agrawal 2014).

Induced responses of plants to herbivory are known to display genetic variation (Gols *et al.* 2008b; Wu *et al.* 2008; Poelman *et al.* 2009; Wason *et al.* 2013). This includes variation in differential regulation of cross-talk between plant molecular defensive pathways under dual herbivore attack (Agrawal *et al.* 2014). As a consequence, plant-mediated effects on community dynamics may vary for plant genetic background, even within species (Johnson *et al.* 2006; Gols *et al.* 2008b; Poelman *et al.* 2009; Uesugi *et al.* 2013). However, very limited information is available on how insects with different feeding modes arriving in various sequences (simultaneous or subsequent) modulate each other's season-long effects on community development and whether these processes are dependent on plant genetic background.

Here, we studied the effects of early herbivores from two different feeding guilds and their order of arrival on arthropod community development on wild perennial *Brassica oleracea* plants (Figure 1) from two different populations (Kimmeridge (KIM) and Winspit (WIN)) over a total period of two years. In a common garden experiment, either aphids (*Brevicoryne brassicae* (A)) or caterpillars (*Plutella xylostella* (C)) or no herbivores (None) were placed on plants of the two *B. oleracea* populations. Second, on another set of plants of the two plant populations, the same species of early-season herbivores were introduced in several different orders of arrival, e.g. aphids followed six days later by caterpillars (A-C), caterpillars followed by aphids (C-A) or both simultaneously (A&C). After inoculation with the herbivores, both sets of plants were monitored throughout the field season at twelve time points for the composition of their associated insect community. In the second year, we focussed on the effect of herbivore arrival order on insect community development. Each plant that had received inoculation with herbivores in different orders of arrival received the same early-season herbivory treatment as in the first year after which the arthropod community was monitored twice during the season for insect colonization.

We specifically addressed the following questions: 1) does the identity of early-season herbivores, either phloem-feeding aphids or leaf-chewing caterpillars, affect season-wide arthropod community development? 2) does the order of arrival of early-season aphids and caterpillars affect arthropod community development? and 3) do early-season herbivores and their order of arrival affect community development differentially on plants of different populations? In other words, is there an interaction effect of early-season herbivore feeding and plant population? To assess which part of the community was most affected by early season herbivory, we analysed both the overall community as well as the community split up in different functional groups of arthropods.

Figure 1. Wild *B. oleracea* plant as grown during the first field season (2012). An individual plant from the population Kimmeridge is shown, in week 31. Photo credits: Jeltje M. Stam.

Materials and Methods

Plants and insects

For the common-garden experiment, wild perennial *Brassica oleracea* L. (Brassicaceae) plants from two plant populations were used. Seeds had been collected from *B. oleracea* populations at the South-Western coast of England, at Winspit (50°35'N, 2°02'W) and Kimmeridge (50°36'N, 2°07'W) (Gols *et al.* 2008b). Seeds were sown directly onto peat soil (Lentse Potgrond No. 4, Lent, The Netherlands), and 11-day-old seedlings were transplanted into peat soil cubes. One week prior to planting, seedlings were placed outside (under a roof shelter) to condition them to field situations. In week 21 (mid-May) 2012, 5-week-old plants were planted directly into the soil of the field site. During the field seasons, plots were manually weeded at regular time intervals and grass strips between plots regularly mown.

After the 2012 field season, the perennial *B. oleracea* plants remained in the field site during winter, and were used next spring for the 2013 field season. The plants were covered with cloth (26 g m^{-2}; AMEVO, the Netherlands) from January 8, 2013 until April 3, 2013 to avoid dehydration of the plants by frost during the coldest part of the winter.

Cabbage aphids, *Brevicoryne brassicae* L. (Hemiptera: Aphididae), and diamondback moth caterpillars, *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), were used as

herbivores to induce the plants early in the season. Both species are specialists on Brassicaceae and occur on the natural cabbage populations (Moyes *et al.* 2000). Both species colonize plants early in the season although not necessarily in a fixed order (Poelman *et al.* 2009; Poelman *et al.* 2010). The insects originated from cultures maintained at the Laboratory of Entomology, Wageningen University, the Netherlands, and were reared on Brussels sprout plants (*B. oleracea* var. *gemmifera* cv Cyrus) under standardized conditions (21±1 °C, 50-70 % relative humidity, 16L : 8D cycle) in a climate chamber.

Monitoring arthropod community – early herbivore identity

To investigate whether the identity of early-season herbivores affected subsequent community development throughout the season, and the role of plant populations in this, we inoculated plants from the Winspit and Kimmeridge populations with *B. brassicae* aphids or *P. xylostella* caterpillars in a common-garden setup. A field site in the vicinity of Wageningen University, Wageningen, the Netherlands, with 48 plots, each with 12 plants in a four by four square (omitting the four central plants to ensure equal plant neighbouring effects for all plants per plot) was established. Each plot was planted with plants from either of the two plant populations, planting distance of 1 m and 4 m wide strips separating the plots, sown with a *Poa/Lolium* mixture directly after planting. Native *Brassica nigra* L. (Brassicaceae) plants, grown and planted similar to the method described for the *B. oleracea* plants, were used to plant an edge (a strip of 1 m wide with 2 rows of plants, 0.5 m distance within-row, at 4 m distance from the plots) surrounding the experimental field, to homogenize edge effects. In week 21 of the second year (2013), new *B. nigra* seedlings were planted in a similar edge around the field site. In week 22 (May 29, 2012) all 12 *B. oleracea* plants in the plot received the same herbivory treatment, consisting of either five adult or 4th instar nymphs of *B. brassicae* aphids (A) or three *P. xylostella* second larval stage (L2) caterpillars (C) or no herbivores (None). These numbers were chosen to resemble natural early-season colonisation (Poelman *et al.* 2008a; Poelman *et al.* 2010), and insects were not removed to mimic the natural colonisation process. For each population, each treatment had eight replicates (plots), which were completely randomized over the 1 ha field site. One week after inoculation, four plants per plot were chosen based on established feeding of the inoculated herbivores and these four plants were monitored throughout the season at 12 time points (week 23 – 41), each time point taking 1 – 2 weeks and at every time point the same four plants were monitored (excluding a few plants that died during the season). During monitoring, each leaf was carefully checked on both sides for all occurring insects and other organisms (e.g. including spiders, snails); excluding fast-flying insects such as butterflies and parasitoids as they could not accurately be assigned to individual plants (see Supplementary Table 1 for list of recorded arthropods). All visible life stages (egg, larva, pupa, adult except when fast flying) were recorded and summed per species.

Monitoring arthropod community – order of early herbivore arrival

To test whether the order of arrival of early-season herbivores and their interaction with plant population affected the subsequent community development over time, another set of 48 plots (eight per treatment for both Winspit and Kimmeridge populations) in the same field site was inoculated with either five *B. brassicae* 4th instar nymphs or adult aphids and three *P. xylostella* L2 caterpillars simultaneously (A&C); five *B. brassicae* aphids, followed six days later by three *P. xylostella* caterpillars (A-C); or three *P. xylostella* caterpillars, followed six days later by five *B. brassicae* aphids (C-A). The first round of herbivore inoculation was in week 22 (May 29, 2012), the second round in week 23 (June 4, 2012), and monitoring of the arthropod community started the day thereafter, as described above. In addition to effects of herbivore order of arrival, the effects of double herbivory compared to single herbivory were examined, by comparing these dual-induced plants in 2012 to plants that had received a single herbivore species in the same year (above).

In the consecutive year we focussed on the effects of order of early herbivore arrival only, and each of the dual-induced plots in the first year received the same herbivore inoculation in the next year, in week 20 (May 16, 2013) and six days later in week 21 (May 22, 2013). Because the perennial *B. oleracea* plants were larger and flowered in 2013, monitoring of the arthropod community was done at two time points: early in the season (round 1, week 21 – 25) and in mid-season (round 2, week 25 – 33), following the same method as in the previous year. In addition, for each insect found, we recorded whether it occurred on the vegetative (leaves) or reproductive (flowers and flower stems) part of the plant, to which we refer as the leaf- and flower-associated communities respectively.

Harvesting seeds

To determine whether the order of early-season herbivore arrival affected seed production in the first reproducing season, as a proxy of plant fitness, all seeds of the monitored *B. oleracea* plants that received both early herbivore species (A&C, A-C, C-A) were harvested after the last round of monitoring. Flower stalks with dried seed pods were cut and placed in a paper bag per plant; a cloth underneath the plant was used to collect all seeds that fell during the procedure. Bag contents were processed after storage to separate seeds from seed pods and other plant material. The numbers of obtained seeds were estimated by weighing 100 seeds per plant and weighing the total plant seed harvest, computing total seed number per plant by dividing total mass by average seed weight.

Statistical analysis

Insect abundance and species richness were assessed per plot in both years. For abundance, numbers of insects per plot were transformed ($x=x_i/x_{max}$) to rescale to

values between 0 and 1, because abundances differed by a factor 10-1,000 between species (Zuur *et al.* 2007), and averaged over the whole season. Species richness was represented by the total number of insect species recorded per plot over the whole season. Effects of early-season herbivory treatment, plant population and their interaction on total herbivore and carnivore abundance and richness in both years were analysed by two-way ANOVA.

Arthropod community composition development during the season was analysed using ordination techniques. Arthropod numbers per species were summed for all monitored plants in a plot and divided by the number of plants monitored in that plot and log (n+0.25) transformed for analysis. *B. brassicae* and *P. xylostella* were excluded from the analyses, because their numbers were directly manipulated during experiments (see supplementary material). To (pairwise) compare effects of treatments and/or plant populations, on the whole arthropod community or on functional groups (herbivores, predators, parasitoids), or plant parts (vegetative or reproductive), subsets of plots or species were selected for analyses. Plant population * early season herbivore treatment interactions were analysed using treatments defined as the plant population-herbivore treatment combination (e.g. WIN A&C).

All ordination analyses were executed with Canoco 5.04 for Windows (ter Braak & Šmilauer 2012).

Field season 2012: early herbivore identity, order of arrival and plant populations

Effects of early-season herbivore identity (aphid or caterpillar), effects of single or dual herbivory and their order of arrival at the two plant populations, on the community composition were analysed using the ordination technique Principle Response Curves (PRC; Šmilauer & Lepš 2014). For each comparison, separate Redundancy Analyses (RDAs) with monitoring time points as covariate were performed using a Monte Carlo permutation test (499 permutations, with hierarchical design for the randomized plots * time points). Resulting PRC plots show relative community composition on the first ordination axis over time, along with a species score plot which indicates the relative species abundance in the community, as affected by the treatments tested. See supplementary material for interpretation of the resulting PRC graphs.

Field season 2013; order of arrival and plant populations

Effects of the order of early-season herbivore arrival on each of the two plant populations on the composition of the arthropod community during the 2013 season was analysed using RDA, a linear constrained ordination technique (Šmilauer & Lepš 2014). Using a linear method was considered valid as the response data had

a gradient of less than 3 turnover (SD) units long. To take into account that each of the two monitoring rounds took several weeks to complete, week number was used as covariate in analyses (i.e. partial RDA). Partial RDAs were performed using a Monte Carlo permutation test (499 unrestricted permutations). Resulting RDA plots show species (arrows) and treatment (square centroids) ordination, with (large) arrows and centroids pointing in the same direction having a high correlation. See supplementary material for interpretation of the resulting RDA biplots.

Seed harvest (2013)

The number of seeds from plants treated with different orders of early herbivore arrival (A&C, A-C, C-A) and the two plant populations (Winspit and Kimmeridge), and their interaction term were analysed by ANOVA. The number of seeds from individually monitored plants was double square root transformed prior to analysis to meet assumptions of normality and homogeneity.

ANOVA's over cumulative *B. brassicae* numbers, insect abundance and species richness, and seed numbers were performed with SPSS Version 19.0.0.1 (Armonk, NY, USA: IBM Corp.).

Results

Arthropod community

Insect abundance and species richness

Both herbivore and carnivore abundance were affected by plant population in the first year, with a higher abundance on Winspit plants, although neither early-season herbivory treatments nor the interaction with plant population affected abundance or species richness in either year (Supplementary Table 2).

Effect of identity of early-season herbivores

By using ordination analyses that provide much more detailed analyses of the community dynamics through time, we identified that early-season herbivory in the first year (2012) had an effect on arthropod community composition during the remainder of the season for both Winspit and Kimmeridge plants (PRC, Winspit plants: first axis explained 5.86%, Monte Carlo permutation test, Pseudo-$F_{60,11}$:31.4; P=0.002, Figure 2a; and on Kimmeridge plants: first axis explained 4.46%, Monte Carlo permutation test, Pseudo-$F_{60,11}$:23.5; P=0.002; Figure 2b). However, plant populations differed in the effects of early herbivory on the arthropod community throughout the season (PRC, first axis explained 1.37%, Monte Carlo permutation test, Pseudo-$F_{12,11}$:15.7, P=0.002), and the effects of plant population and early-season herbivore inoculation showed a significant interaction (PRC, first axis

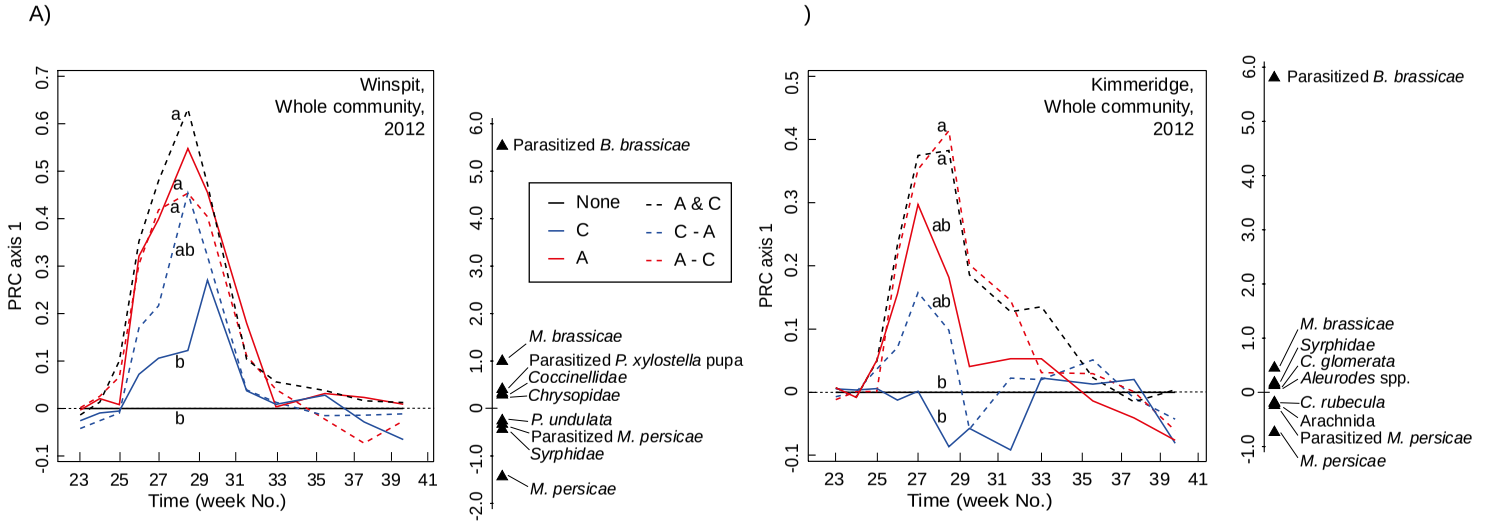

Figure 2. Principal Response Curves (PRC) showing the whole community development throughout the season. Shown is the first PRC axis over time for a) Winspit plants (WIN) and b) Kimmeridge plants (KIM), and the species scores on the first ordination axis. Abundance of species high on this species score plot correlate with treatments with a high score on the first principle component axis for community composition, and *vice versa*. Only the 9 most important species (most extreme (highest/lowest) ordination scores) are depicted, excluding *B. brassicae* and *P. xylostella* because their numbers are directly affected by experimental manipulation. Different letters denote significant difference between treatments on the response curve after pairwise comparisons. Early season herbivory treatments: None: no early season herbivore induction, set as baseline; C: caterpillar *P. xylostella*; A: aphid *B. brassicae*; A&C: aphid & caterpillar simultaneously; C-A: caterpillars, 6 days later followed by aphids; A-C: aphids, 6 days later followed by caterpillars. Cumulative explained variation of the first four principal component axes: 10.78% (WIN); 8.38% (KIM).

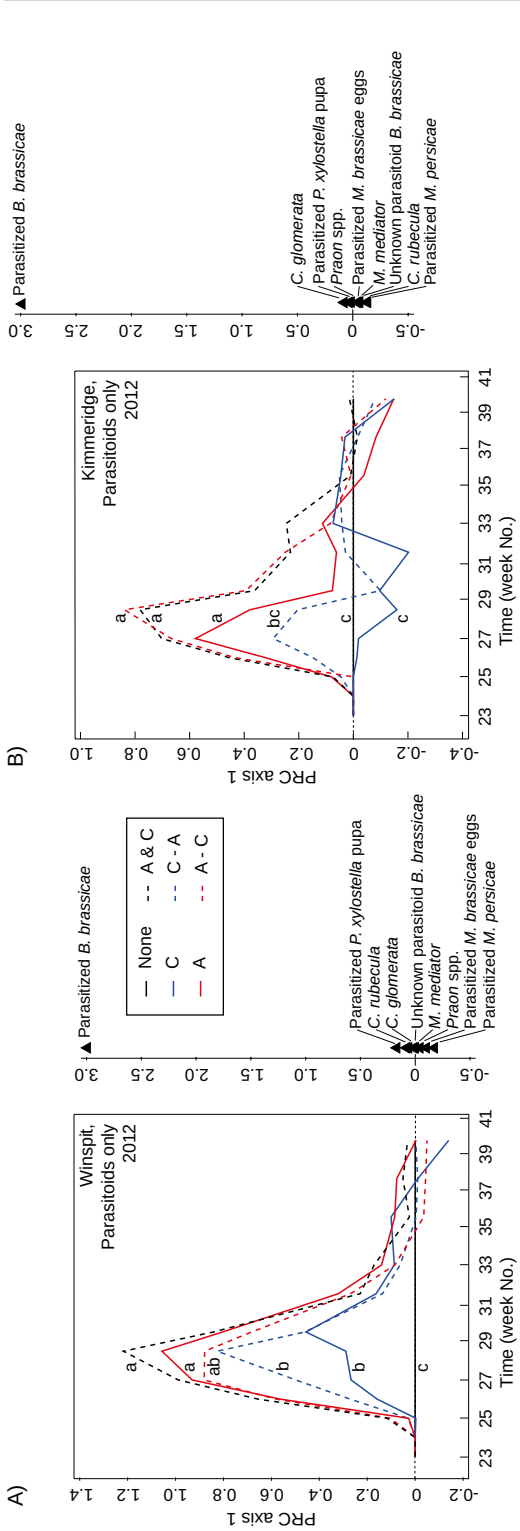

Figure 3. Principal Response Curves (PRC) showing the parasitoid community development throughout the season. Shown is the first PRC axis over time for a) Winspit plants (WIN) and b) Kimmeridge plants (KIM), and the species scores on the first ordination axis. Abundance of species high on this species score plot correlate with treatments with a high score on the first principle component axis for community composition, and *vice versa*. All 9 parasitoid species recorded are depicted. Different letters denote significant difference between treatments on the response curve after pairwise comparisons. Early season herbivory treatments: None: no early season herbivore induction, set as baseline; C: caterpillar *P. xylostella*; A: aphid *B. brassicae*; A&C: aphid & caterpillar simultaneously; C-A: caterpillars, 6 days later followed by aphids; A-C: aphids, 6 days later followed by caterpillars. Cumulative explained variation of the first four principal component axes: 22.72% (WIN); 19.05% (KIM).

Table 1. Results of principal component analyses (PCA; a redundancy analysis with time component) on the arthropod community on plants induced with one or two herbivores in different orders of arrival. Early season herbivore induction treatments (None, A, C, A&C, A-C, C-A) on two plant populations Kimmeridge (KIM) and Winspit (WIN) were tested in 2012 on different parts of the community with Monte Carlo permutation tests. [a] Percentages show the % explained variation on the first RDA axis; [b] d.f. (i,j) show the degrees of freedom of explanatory variables (i) and covariates (j). [c] F-values are pseudo-F values of Monte Carlo Permutation test. [d] parasitoids associated with the aphid *B. brassica* ('mummies') were excluded from the community analysis.

Part of community	Treatments tested	Plant population	% [a]	d.f. [b]	F [c]	P-value
Single early season herbivores						
All arthropods	A vs None	WIN	8.48	12,11	15.6	**0.004**
All arthropods	C vs None	WIN	2.18	12,11	3.7	0.538
All arthropods	A vs C	WIN	4.80	12,11	8.5	**0.016**
All arthropods	A vs None	KIM	3.29	12,11	5.7	0.140
All arthropods	C vs None	KIM	1.53	12,11	2.6	0.890
All arthropods	A vs C	KIM	3.25	12,11	5.6	0.130
Herbivores only	All treatments	WIN	4.51	60,11	23.8	0.580
Herbivores only	All treatments	KIM	2.74	60,11	14.2	0.974
Predators only	All treatments	WIN	3.64	60,11	19.1	0.570
Predators only	All treatments	KIM	3.43	60,11	17.9	0.776
Parasitoids only	A vs None	WIN	30.52	12,11	73.8	**0.004**
Parasitoids only	C vs None	WIN	5.49	12,11	9.8	**0.050**
Parasitoids only	A vs C	WIN	12.89	12,11	24.9	**0.018**
Parasitoids only	A vs None	KIM	8.64	12,11	15.9	**0.014**
Parasitoids only	C vs None	KIM	2.81	12,11	4.9	0.442
Parasitoids only	A vs C	KIM	10.49	12,11	19.7	**0.004**
Single vs double early season herbivores						
All arthropods	C vs A&C	WIN	6.19	12,11	11.1	**0.002**
All arthropods	C vs C-A	WIN	2.84	12,11	4.9	0.230
All arthropods	C vs A&C	KIM	6.95	12,11	12.5	**0.004**
All arthropods	C vs C-A	KIM	2.05	12,11	3.5	0.584
All arthropods	A vs A&C	WIN	2.07	12,11	3.5	0.592
All arthropods	A vs A-C	WIN	3.08	12,11	5.3	0.122
All arthropods	A vs A&C	KIM	2.44	12,11	4.2	0.452
All arthropods	A vs A-C	KIM	2.70	12,11	4.7	0.236

Parasitoids only	C vs A&C	WIN	14.16	12,11	27.7	**0.002**
Parasitoids only	C vs C-A	WIN	4.23	12,11	7.4	0.232
Parasitoids only	C vs A&C	KIM	21.33	12,11	45.6	**0.002**
Parasitoids only	C vs C-A	KIM	3.98	12,11	7.0	0.264
Parasitoids only	A vs A&C	WIN	2.36	12,11	4.1	0.610
Parasitoids only	A vs A-C	WIN	1.69	12,11	2.9	0.932
Parasitoids only	A vs A&C	KIM	4.95	12,11	8.8	0.192
Parasitoids only	A vs A-C	KIM	6.44	12,11	11.6	0.064
Double early season herbivores						
All arthropods	A&C, A-C, C-A	WIN	2.26	24,11	5.8	0.508
All arthropods	A&C, A-C, C-A	KIM	3.24	24,11	8.4	0.080
Herbivores only	A&C, A-C, C-A	WIN	3.23	24,11	8.4	0.680
Herbivores only	A&C, A-C, C-A	KIM	2.12	24,11	5.5	0.960
Predators only	A&C, A-C, C-A	WIN	3.90	24,11	10.2	0.300
Predators only	A&C, A-C, C-A	KIM	3.76	24,11	9.8	0.366
Parasitoids only	A&C, A-C, C-A	WIN	6.32	24,11	17.0	**0.036**
Parasitoids only	A&C vs C-A	WIN	8.08	12,11	14.8	**0.004**
Parasitoids only	A&C vs A-C	WIN	4.14	12,11	7.3	0.236
Parasitoids only	C-A vs A-C	WIN	4.63	12,11	8.2	0.196
Parasitoids only	A&C, A-C, C-A	KIM	9.52	24,11	26.5	**0.004**
Parasitoids only	A&C vs C-A	KIM	9.84	12,11	18.3	**0.010**
Parasitoids only	A&C vs A-C	KIM	1.73	12,11	3.0	0.892
Parasitoids only	C-A vs A-C	KIM	10.47	12,11	19.7	**0.002**
Parasitoids only, excluding *Bb.* parasitoids [d]	All treatments	WIN	3.52	60,11	18.4	0.434
Parasitoids only, excluding *Bb.* parasitoids [d]	All treatments	KIM	3.46	60,11	18.0	0.480

explained 5.48%, Monte Carlo permutation test, Pseudo-$F_{60,11}$: 29.2, P=0.002). Therefore, the plant populations were analysed separately for the remainder of this study.

When comparing the effect of early-season herbivore identities, aphids (A) placed on Winspit plants early in the season differentially affected season-wide development of the plant-associated insect community, compared to community development

on plants that had an early-season caterpillar treatment (C), or compared to plants without early-season herbivore inoculation (None) (Table 1, Figure 2a). In contrast, on Kimmeridge plants early-season herbivory by aphids or caterpillars did not significantly affect the insect community (Table 1, Figure 2b).

When the responding arthropod community was divided into different functional groups (herbivores, predators, parasitoids, see Supplementary Table 1), early-season herbivore identity only affected the parasitoid community, but not the herbivore and predator communities (Table 1). Aphid inoculation on both plant populations predominantly increased the abundance of parasitoids associated with *B. brassicae*, compared to plants with caterpillar inoculation or no herbivores (see species score plots in Figures 2 and 3, Table 1).

Effects of single versus double early-season herbivores

In 2012, there were plots treated with either single herbivores (A, C or None) or with dual herbivores (A&C, A-C, C-A); dual infestation had a different effect on the arthropod community than infestation with a single herbivore species. Caterpillars alone (C) resulted in fewer parasitized *B. brassicae* (mummified aphids) on both plant populations compared to plants with simultaneous caterpillar and aphid inoculation (C&A) or with aphids followed by caterpillars (A-C), although not when compared to plants inoculated with caterpillars followed by aphids (C-A) (Figure 2a,b, Table 1). When aphids were present on the plant, however, it did not matter for the arthropod community whether they were alone (A), or together with caterpillars (A&C) or followed by caterpillars (A-C; Figure 2, Table 1). These results were similar when only the responding parasitoid community was considered, but effects of single or double herbivory did not differ from each other for the herbivore or predator community (Figure 3a,b, Table 1).

Effects of order of arrival of early-season herbivores

Since dual herbivory differed from single herbivory, we addressed whether the order of arrival of herbivores in the dual herbivory treatments modulated the development of the insect community differently. In both seasons, the insect community development was specific for the combination of effects of the order of arrival of the two early-season herbivores and the plant population on which they were found (significant double herbivore inoculation treatment * plant population interaction; 2012: PRC, first axis explained 3.78%, Monte Carlo permutation test, Pseudo-$F_{60,11}$: 19.8, P=0.016; 2013: Table 2, Figure 4). In 2012, the community composition differed between the two plant populations (PRC, first axis explained 1,79%, Monte Carlo permutation test, Pseudo-$F_{12,11}$: 10.1, P=0.004), whereas this was not the case in 2013 (Table 2). The order of early-season herbivore arrival itself did not result in significant effects on community development in either of the two years (Figure 2, Tables 1 and

Table 2. Results of Partial Redundancy analyses (partial RDA) on the arthropod community on plants induced with two herbivores in different orders of arrival. Early-season double herbivore inductions (A&C, A-C, C-A) in 2013 on two plant populations Kimmeridge (KIM) and Winspit (WIN), and their interaction were tested for significant differences. Values in **bold** indicate significant effects. 'Induction + Plant population' explores the effects of both, but without their interaction effect. R1: data of the first round of observations in the field (week 21-25); R2: data of the second round of observations in the field (week 25-33). [a] Percentages show the % explained variation on the first RDA axis; [b] F-values are pseudo-F values of Monte Carlo Permutation test with subscripts of F-values (i,j) show the degrees of freedom (df) of explanatory variables (i) and covariates (j). Carnivores comprise of both predators and parasitoids.

Part of community	Monitored rounds	Factors tested											
		Induction			**Plant Population**			**Induction + Plant population**			**Induction * Plant population**		
		%[a]	**F[b]**	**P**	**%**	**F**	**P**	**%**	**F**	**P**	**%**	**F**	**P**
All arthropods	**R1+R2**	1.70	$F_{2,10}$=1.2	0.224	1.68	$F_{1,10}$=1.4	0.190	1.91	$F_{3,10}$=1.2	0.162	3.63	$F_{5,10}$=1.4	**0.024**
Herbivores only	**R1+R2**	1.63	$F_{2,10}$=1.0	0.446	2.21	$F_{1,10}$=1.8	0.094	3.00	$F_{3,10}$=1.2	0.234	5.33	$F_{5,10}$=1.7	**0.024**
Herbivores only	**R1**							3.65	$F_{3,4}$=0.9	0.532	9.81	$F_{5,4}$=1.7	**0.028**
Herbivores only	**R2**							4.08	$F_{3,6}$=1.0	0.414	7.89	$F_{5,6}$=1.1	0.296
Predators only	**R1+R2**							2.06	$F_{3,10}$=1.0	0.460	3.25	$F_{5,10}$=1.1	0.334
Parasitoids only	**R1+R2**							4.25	$F_{3,10}$=1.4	0.204	5.08	$F_{5,10}$=1.0	0.366
Carnivores only	**R1+R2**							2.96	$F_{3,10}$=1.2	0.234	3.78	$F_{5,10}$=1.1	0.354
Carnivores only	**R1**							2.63	$F_{3,4}$=0.6	0.898	6.30	$F_{5,4}$=1.0	0.482
Carnivores only	**R2**							6.79	$F_{3,6}$=1.4	0.164	11.51	$F_{5,6}$=1.3	0.162
On flowers	**R1+R2**	4.37	$F_{2,10}$=2.1	**0.032**	2.10	$F_{1,10}$=1.7	0.124	4.87	$F_{3,10}$=2.1	**0.016**	6.05	$F_{5,10}$=1.7	**0.022**
On flowers	**R1**							3.71	$F_{3,4}$=0.8	0.744	10.83	$F_{5,4}$=1.4	0.112
On flowers	**R2**							9.11	$F_{3,6}$=1.8	0.072	14.75	$F_{5,6}$=1.7	0.058
On leaves	**R1+R2**	1.16	$F_{2,10}$=1.8	0.686	1.70	$F_{1,10}$=1.4	0.180	1.98	$F_{3,10}$=1.0	0.456	3.37	$F_{5,10}$=1.3	0.112
On leaves	**R1**							3.91	$F_{3,4}$=1.0	0.426	10.50	$F_{5,4}$=1.7	**0.016**
On leaves	**R2**							3.09	$F_{3,6}$=1.1	0.372	4.95	$F_{5,6}$=0.9	0.578

2). The course of development of the insect community thus strongly depended on the interaction between the plant populations and order of arrival of herbivores (Supplementary Table 3 and Supplementary material). For example, inoculation of plants with aphids followed by caterpillars (A-C) resulted in larger numbers of the generalist aphid *Myzus persicae* on Kimmeridge plants, but on Winspit plants with the same order of herbivores the effect was opposite, resulting in fewer *M. persicae* (Figure 4, species arrow for *M. persicae* is large in the direction of KIM A-C, but points away from WIN A-C).

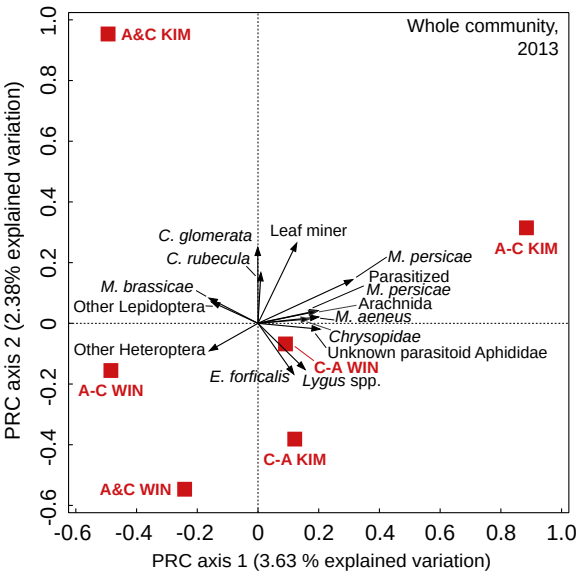

Figure 4. Partial Redundancy Analysis (partial RDA) showing the ordination of species in the community and the order of early herbivore arrival*plant population interactions. Shown are the first two ordination axes with squares indicating factors (order of early-season herbivore arrival * plant population interaction), and vector arrows indicating species ordination. Data of both monitoring rounds of the 2013 season are used. The 15 most important (longest arrows) species are shown, except *B. brassicae* and *P. xylostella* because their numbers are directly affected by experimental manipulation. Cumulative explained variation of the first four ordination axes: 8.19%.

Separate analyses of the effects on different functional groups (herbivores, predators, parasitoids, see Supplementary Table 1) further revealed the specificity of community responses to induction by early herbivores in different orders of arrival on the two plant populations. In 2012, only the parasitoid community was affected by the interaction between herbivore induction and plant population (PRC, first axis explained 10.71%, Monte Carlo permutation test, Pseudo-$F_{60,11}$: 60.5, P=0.002, Table 2, Figure 3). To illustrate this, on Kimmeridge plants significantly more parasitized *B. brassicae* were found on plants first induced by aphids (A-C) than on plants first induced by caterpillars (C-A). In contrast, on Winspit plants, the order of arrival (C-A vs A-C) did not affect the parasitoid community.

In 2013, however, the specificity in interactions between order of herbivore arrival and plant populations was found for the response of the herbivore community but not the predator or parasitoid communities (Table 2; Supplementary Table 4 and Supplementary material).

Time since inoculation also affected the response of the herbivore community, and this was different for the two plant populations and order of early herbivore combinations. In the first half of the season in 2013, for example, the larvae of the specialist moth *Evergestis forficalis* were more abundant on Kimmeridge plants inoculated with caterpillars followed by aphids (C-A) than on Winspit plants with the same treatment (C-A) (Supplementary Figure 1). Later in the season this effect disappeared (Table 2: no significant effect of treatments on herbivores in round 2).

Early season herbivores and effect on leaf- or flower-associated insects

In 2013, the perennial *B. oleracea* plants flowered and developed seeds during the season. The arthropod communities on the vegetative (leaves) or on the reproductive (flowers) parts of the plants were differentially affected by the order of herbivore arrival. Moreover, these effects were different for the two plant populations. The flower-associated community was affected by the order of arrival of early-season herbivores that had been introduced on the leaves (Table 2). To illustrate this, pair-wise comparisons showed that the community composition on flowers differed on plants induced with caterpillars followed by aphids (C-A), compared to plants on which aphids and caterpillars had been introduced simultaneously (A&C) (PCA, first axis explained 5.93%, Monte Carlo permutation test, Pseudo-$F_{1,10}$: 3.2; P=0.022; while A&C vs A-C: PCA, first axis explained 4.08%, Monte Carlo permutation test, Pseudo-$F_{1,10}$:2.0, P=0.082; and A-C vs C-A: PCA, first axis explained 1.73%, Monte Carlo permutation test, Pseudo-$F_{1,10}$:0.9, P=0.452). The response of the flower-associated community was also specific for both the order of herbivore arrival and the plant population (significant interaction effect between the two factors; Supplementary Table 5), even though the communities did not differ between the plant populations (Table 2).

In contrast, the leaf-associated arthropod community was not affected by the order of introduction of early season herbivores, the plant populations on which they were introduced, or the interaction between the two (Table 2). Thus, in 2013 the flower-associated community, compared to the leaf-associated community, was more responsive to plant genetic background and differences in early season herbivores on those plants, even though early season herbivores were initially inoculated on the leaves.

Seed set (2013)

The number of seeds collected in 2013 was not affected by the order of arrival of early herbivores (A&C, A-C, C-A; ANOVA F_2:0.126, P=0.882) or the plant population (ANOVA F_1:1.252, P=0.265), but a near-significant interaction effect between the two factors was observed (ANOVA F_2:2.952, P=0.055; Supplementary Figure 2).

Discussion

Plant-mediated species interactions profoundly structure insect communities (van Zandt & Agrawal 2004b; Kessler & Halitschke 2007; Viswanathan *et al.* 2007; Stam *et al.* 2014; Utsumi 2015). Here, we found that early-season feeding by either of two herbivores that belong to different feeding guilds resulted in a different composition of the arthropod communities associated with these plants. The effects of early herbivores were found to be non-additive, as feeding by the two early-season herbivores from the same plant differentiated insect community dynamics from effects caused by each herbivore individually. The order of arrival of the two inducers further differentially affected community composition, indicating this is an important component driving community development. Moreover, order of early-season herbivore arrival affected the community composition in a specific manner, depending on the plant population. Our study remained inconclusive on the importance of these effects for plant fitness as the seed set was only nearly significantly different for the interaction term of plant population and herbivore treatment. Both density- and trait-mediated processes may have structured communities on dual-herbivore-induced plants. Aphids and caterpillars are known to differentially induce plant traits ranging from differential transcriptomic effects (de Vos *et al.* 2005; Bidart-Bouzat & Kliebenstein 2011) to differential phenotypes (Soler *et al.* 2012a; Rowen & Kaplan 2016). While density-dependent effects of *B. brassicae* aphid arrival caused later higher abundance of *B. brassicae*-associated parasitoids in 2012 (Křivan & Schmitz 2004), indirect plant-trait mediated interactions likely played a role in the effects of early-herbivore arrival on changes in herbivore abundance in 2013 (Utsumi *et al.* 2010). The density-dependent effects of aphids on the emission of aphid-induced plant volatiles may have played a role in density-dependent effects on the attraction of parasitoid of aphids as well as parasitoids that attack other herbivore species (Ponzio *et al.* 2016). In our experiments, the early-season herbivores that we had placed on the plants were not removed and thus, direct effects of these on subsequent community members cannot be excluded. Yet, the ample evidence on herbivore-induced changes in plant traits, both at the site of herbivory as well as systemically throughout the plant, and their consequences for interactions with the members of the plant-associated insect community (Stam *et al.* 2014) support an important role for trait-mediated effects. These include among others herbivore induced changes in secondary metabolites such as glucosinolates, plant volatiles, plant growth and physical plant characteristics (Kessler & Baldwin 2002; Howe & Jander 2008; Stam *et al.* 2014).

Identity and order of arrival

Identity of early herbivores as a factor influencing the induction of plant responses and their effects on the development of plant-associated insect communities was

previously also found for leaf-chewing herbivores on milkweed plants (*Asclepias syriaca*) (van Zandt & Agrawal 2004b). Herbivores of different feeding guilds induce widely different plant responses (van Zandt & Agrawal 2004a; de Vos *et al.* 2005; Rodriguez-Saona *et al.* 2010; Bidart-Bouzat & Kliebenstein 2011) in terms of for example plant quality for herbivores or induction of volatiles (Stam *et al.* 2014). This may explain the differential effects of early aphid or caterpillar colonizers on subsequent community development in our study (Poelman *et al.* 2008a; Soler *et al.* 2012a; Stam *et al.* 2014). Moreover, multiple herbivory or single-herbivore infestation result in different plant responses, especially when those herbivores are of different feeding guilds (Zhang *et al.* 2009; Soler *et al.* 2012a; Ali & Agrawal 2014), or arrive in different sequences (Voelckel & Baldwin 2004; Erb *et al.* 2011). SA-inducing aphids may interfere with plant responses to JA-inducing caterpillars with consequence for plant phenotype, but the reverse effects are less pronounced (Zarate *et al.* 2007; Vos *et al.* 2013a). Here, we indeed found that community responses to early-season herbivores were comparable when aphids and caterpillars were present together (A&C) or when aphids were alone (A) or followed by caterpillars (A-C), and were different from a situation where caterpillars were introduced first (C-A). The order of caterpillar and aphid arrival thus shaped community dynamics in a manner that is predicted on the basis of cross-talk between SA and JA pathways (Thaler *et al.* 2012a). Future studies should however establish whether cross-talk is causal to the effect of herbivore order of arrival on community development.

Importantly, our data suggest that the first-arriving herbivore had the strongest effect on the further development of the insect community. When comparing only leaf chewers, other studies found that the first-arriving herbivore strongly determines plant resistance to other herbivores (Viswanathan *et al.* 2007), although subsequent herbivores have been found to override effects by first herbivores as well (van Zandt & Agrawal 2004b; Erb *et al.* 2011; Miller-Pierce & Preisser 2012; Stam *et al.* 2014). The first herbivore may strongly influence community dynamics on a focal plant also because other arthropod species respond differently to the same induced changes in plant traits (van Zandt & Agrawal 2004a; Uesugi *et al.* 2013; Li *et al.* 2014). The induced response to the first herbivore thus affects the likelihood of subsequent arrival of other species. Moreover, the induced plant response to the newly arriving herbivore will build on or interact with the physiological plant response caused by the previous inducer (Underwood 2012). This potentially amplifies effects of differential plant-trait induction by sequential arrival of multiple herbivores, to following interactions in the cascade (Utsumi *et al.* 2010). Thus, the effects of an initial herbivore arrival may initiate trait-mediated effects that affect the colonisation of subsequent herbivores (Poelman *et al.* 2010) with consequences for additional colonisation events and thus may influence community dynamics (Stam *et al.* 2014). Our data do not give exclusive answers to the debate to which herbivores in a sequence most strongly determine subsequent herbivore arrival (so called keystone herbivores; Poelman & Kessler 2016). In

the 2012 field season, we saw canalizing effects of the first arriving herbivore to community composition with especially aphids having a strong inducing effect. These processes are comparable to those identified by Viswanathan et al. (2007) on *Solanum dulcamara* plants. Apparently *B. brassicae* aphids as early arrivals are keystone determinants of community assembly or so-called community phenotype in our *B. oleracea* system (Whitham *et al.* 2006; Newton *et al.* 2010), either by being most abundant, as is the case here, or because they interact with arthropods that have a large impact on other community members or indirectly affect other arthropods mediated through modified plant traits (Trussell *et al.* 2003; Kessler & Baldwin 2004; Poelman & Kessler 2016). Such key-member-mediated effects have been identified in various systems (Paine 1966; Beschta & Ripple 2015). On the other hand, in 2013 the order of herbivore arrival and plant population could not be seen separate from each other, indicating specificity of a plant response (Miller-Pierce & Preisser 2012; Stam *et al.* 2014) in which both insect presence, their order of arrival and plant population were important.

Specificity of plant populations

Effects of early-season herbivores and the order of their arrival were specific for the plant population on which the interactions occurred. This may be due to variation in inducibility of traits that mediate indirect insect interactions between the populations of wild *B. oleracea* plants, such as the foliar concentration of glucosinolates (Gols *et al.* 2008b; Newton *et al.* 2009b) and the effect size of induced traits on insect community members (Li *et al.* 2014). Moreover, closely related plant species have been found to differ in how they physiologically cope with multiple attackers, suggesting that plant-mediated interactions between herbivores are also a selective force on plant defence strategies (Agrawal *et al.* 2014; Ali & Agrawal 2014; Poelman & Kessler 2016). Here, we found a significant interaction between plant population and effect of the order of herbivore arrival. This suggests that Kimmeridge and Winspit plants differ in SA-JA crosstalk (Kroes et al. 2016), which may result from selection by insect communities that differ in the order of herbivore arrival, or presence/absence of keystone species that subsequently affect a cascade of plant-mediated interactions (Keith *et al.* 2010; Utsumi *et al.* 2013; Stam *et al.* 2014; Poelman & Kessler 2016).

Changes over time and plant ontogeny

Inoculation with herbivores on the leaves had consequences for the flower-associated community, not for the leaf-associated community. This could be due to the physical movement of insects from leaves towards flowers as the season progressed; because systemic induction of phenotypic changes occurs not only in the leaves, but also in the reproductive parts (McCall & Irwin 2006; Kessler &

Halitschke 2009; Lucas-Barbosa *et al.* 2011); or because effects of induced plant phenotypic changes cascaded via consecutive community members up until species that are associated with flowers in a spatial-temporal manner (Utsumi *et al.* 2010). According to the optimal defence theory, plant defence and responsiveness to herbivory should be highest in plant tissues with a high priority for plant fitness (McCall & Fordyce 2010), which might explain the significant effect of order of early season herbivore arrival cascading onto the flower-associated community. Plant ontogenetic variation in (inducible) resistance traits throughout the season or years can be another reason for different patterns during the season (Boege & Marquis 2005). We recorded that during the first part of the season, order of early herbivory and plant population had the most profound effects on the herbivore or leaf-associated community composition, after which effects declined (Figure 2 and 3 and Table 2). A similar seasonal pattern was seen in another study with *B. oleracea* plants (Li et al. 2016), although others found season-long effects of early season herbivory (Viswanathan *et al.* 2005; Poelman *et al.* 2010; Utsumi 2015). Plant ontogeny may affect plant defence and/or tolerance to herbivores (Boege & Marquis 2005), and especially in a perennial system as studied here, plant age can have large effects on insect-plant interactions (Lawrence *et al.* 2003; Quintero & Bowers 2011). Thus, early-season herbivory inducing plant defences first in the leaves, can affect the arthropod community later in the season on the flowers and seeds through a cascading chain of induced plant trait-mediated interactions over time and space (Utsumi *et al.* 2010; McArt *et al.* 2013).

Future directions

Our work shows that the order of herbivore arrival as part of insect community dynamics is a determinant of future season-wide insect attack to plants and that these processes are driven by plant-mediated species interactions. However, not all community members were equally responsive to the inducing herbivores and the inducers themselves differed in how strongly they determined community dynamics. To understand how plants deal with multi-herbivore attack and which physiological adaptations such as the balance of JA-SA crosstalk have evolved in this context, it is important to identify which community members shape the majority of interactions in a plant-trait mediated interaction network (Poelman 2015). Also, plant-mediated insect interactions should be seen in a dynamic community context (Poelman 2015) and it should be evaluated how several (parallel) interaction chains affect each other synergistically or antagonistically.

In addition to variation in community dynamics over the season or variation in underlying mechanisms between years, we know relatively little how inducible plant resistance traits are regulated within the plant over time, and how time lags between inducers play a role in prioritizing or conveying responses to attackers (Utsumi *et al.* 2010; Karban 2011; Underwood 2012). Integrating inducible plant

responses depending on plant genotype with community interactions that occur at very different time scales is a major challenge (Barah & Bones 2014; Stam *et al.* 2014). However, we need to understand both processes to unravel how multiple herbivory influences community structure and which consequences induced responses have on plant fitness (Karban 2011; Poelman & Kessler 2016). Moreover, these eco-evolutionary dynamics may be reflected in trait evolution of other community members (Utsumi *et al.* 2013; Poelman & Kessler 2016).

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Chapter 4

Supplementary material

Brevicoryne brassicae and *Plutella xylostella* numbers in the community

Brevicoryne brassicae aphids and *P. xylostella* caterpillars were placed on the plants early in the season, thereby directly manipulating their numbers. To test whether their numbers on those plants indeed were changed, season-wide cumulative *B. brassicae* numbers (total over all 12 time points in 2012) of *B. brassicae* aphids per plot for the treatments A, C and None for each of the two plant populations were analysed by ANOVA.

The number of *B. brassicae* aphids did not differ between the herbivory treatments. However, a marginally non-significant effect (ANOVA F_2:3.096, P=0.066) of aphid inoculation causing more *B. brassicae* was recorded on Winspit plants. Because their numbers were directly affected by the experimental manipulation, and thus direct density effects and indirect interaction effects could not be separated, *B. brassicae* and *P. xylostella* were left out of further community composition analyses.

Interpretation of ordination plots

Ordination analyses of community composition in field season 2012 resulted in PRC graphs, that show the first ordination axis against time and the ordination scores of each species as projected on the first ordination axis. A high line in the graph (high PRC score for a treatment at a certain time point), correlates with a larger abundance of species scoring high on the species axis; species with a negative score correlate with lower abundance of this species compared to the average abundance on plants with the treatment set as baseline (ordination score = 0).

Ordination analyses of community composition in field season 2013 resulted in RDA biplots, that show on the first two ordination axes the ordination of species (arrows from origin to their ordination on [axis 1, axis 2]), and the ordination of the plant population-early herbivore treatment combinations (WIN A&C, WIN A-C, WIN C-A, KIM A&C, KIM A-C, KIM C-A), which are the centroids of the ordination scores of all plots of the same treatment. RDA graphs can be interpreted using the biplot rule (Šmilauer & Lepš 2014), in which the position of a species in the ordination graph is projected with right angles to the (imaginary) line through the treatment's position and the origin of the ordination graph. Species thus projected far away from the origin are predicted to have a large abundance on plots with that treatment (if they are on the same side of the origin), or a lower than average abundance (if they are on opposite sides of the origin). Plants with treatments with an ordination position close to each other (low Euclidean distance) are predicated to have similar arthropod community compositions.

Herbivore induction * plant population specificity

The specificity of community changes to early herbivore arrival on the different plant populations was apparent from the RDA biplot results, but we further elucidated it with pairwise comparisons of the herbivore induction*plant population combinations using case scores on the first three principal component axes of the community composition in 2013. Therefore, unconstrained linear Principal Component Analyses (PCA, ordination of the field plots without prior knowledge on applied treatments) with week number as covariate were performed. Thus obtained ordination scores on each of the first three ordination axes of each field plot were used for ANOVA analyses on 'treatments', formulated as the plant population*order of herbivore arrival combinations, and round monitored, and their interaction term. A forward accumulated Wald test (normal distribution) with estimated dispersion parameter was performed, followed by an LSD post-hoc test to assess significant differences between all 'treatment' pairs (plant population * early herbivore interaction) if the main effect was significant. ANOVA's over PCA plot scores were performed with GenStat Version 17 (VSN International Ltd, Hemel Hempstead, UK).

These tests between plant population * herbivore order combinations showed that effects of the two factors on the responding community could not be separated, as both pair-wise differences between herbivore sequences (e.g. a significant difference between A&C WIN vs C-A WIN), between plant populations (e.g. A-C KIM vs A-C WIN), and across both herbivore induction and plant population (e.g. A&C WIN vs A-C KIM) were significant (Supplementary Table 2).

Also when the responding community was divided into functional groups, herbivores responded very specific to the herbivore induction * plant population interaction: using case scores of the herbivore community ordination, these two factors could similarly not be separated (Supplementary Table 3). This indicates that plant populations differ in their response to early season herbivores and the order of herbivore species arrival, affecting the development of the subsequent herbivore community on those plants.

Supplementary Table 1. Arthropod species found during field monitoring in 2012 and 2013, including their functional group (herbivore / predator / parasitoid). [a] Species was only encountered during 2013 field season; [b] Species was only recorded for the experiments described in Chapter 6. [c] Only cocoons of the species were recorded; [d] Only mummified aphids were recorded.

Species	Order	Family	Functional group	Remark
Phyllotreta undulata	Coleoptera	Chrysomelidae	Herbivore	
Phyllotreta atra	Coleoptera	Chrysomelidae	Herbivore	
Coccinella spp.	Coleoptera	Coccinellidae	Predator	
Meligethes aeneus	Coleoptera	Nitidulidae	Herbivore	
Ceutorhynchus assimilis	Coleoptera	Curculionidae	Herbivore	
Cantharis spp.	Coleoptera	Cantharidae	Predator	2013 only[a]
Amphimallon solstitiale	Coleoptera	Scarabaeidae	Herbivore	2013 only
Unknown spp. of tortoise beetle	Coleoptera	Chrysomelidae	Herbivore	
Pieris rapae	Lepidoptera	Pieridae	Herbivore	
Pieris brassicae	Lepidoptera	Pieridae	Herbivore	
Plutella xylostella	Lepidoptera	Yponomeutidae	Herbivore	
Mamestra brassicae	Lepidoptera	Noctuidae	Herbivore	
Lacanobia suasa	Lepidoptera	Noctuidae	Herbivore	
Autographa gamma	Lepidoptera	Noctuidae	Herbivore	
Evergestis forficalis	Lepidoptera	Crambidae	Herbivore	
Several other species of *Lepidoptera*	Lepidoptera	-	Herbivore	2013 only; Ch 6 only[b]
Brevicoryne brassicae	Hemiptera	Aphididae	Herbivore	
Myzus persicae	Hemiptera	Aphididae	Herbivore	
Other aphids than *B. brassicae* or *M. persicae*	Hemiptera	Aphididae	Herbivore	Ch 6 only
Philaenus spp.	Hemiptera	Aphrophoridae	Herbivore	2013 only
Orius insidiosus	Hemiptera	Anthocoridae	Predator	
Lygus spp.	Hemiptera	Miridae	Herbivore	
Eurydema oleracea	Hemiptera	Pentatomidae	Herbivore	
Aleyrodes spp.	Hemiptera	Aleyrodidae	Herbivore	

Several species of *Symphyta* (sawfly) larvae	Hymenoptera	-	Herbivore	
Cotesia rubecula	Hymenoptera	Braconidae	Parasitoid	Cocoons[c]
Cotesia glomerata	Hymenoptera	Braconidae	Parasitoid	Cocoons
Microplitis mediator	Hymenoptera	Braconidae	Parasitoid	Cocoons
Praon spp.	Hymenoptera	Braconidae	Parasitoid	Cocoons
Species parasitizing *P. xylostella* (likely *Diadegma* spp.)	Hymenoptera	Ichneumonidae	Parasitoid	Cocoons
Several species parasitizing *B. brassicae*	Hymenoptera	-	Parasitoid	Mummies[d]
Several species parasitizing *M. persicae*	Hymenoptera	-	Parasitoid	Mummies; Ch 6 only
Several species parasitizing other aphids than *B. brassicae* or *M. persicae*	Hymenoptera	-	Parasitoid	Mummies; Ch 6 only
Several species of Syrphidae	Diptera	Syrphidae	Predator	
Unknown species of gall midge larvae	Diptera	Cecidomyiidae	Predator	
Unknown species of thrips	Thysanoptera	-	Herbivore	
Several species of Neuroptera	Neuroptera	-	Predator	
Several species of spiders	Araneae	-	Predator	
Several species of snails and slugs	-	-	Herbivore	Class Gastropoda
Several (unknown) species of leaf mining insects	-	-	Herbivore	2013 only

Supplementary Table 2. ANOVAs of arthropod abundance and species richness affected by early season herbivore treatment, plant population and their interaction in both years. Numbers in **bold** indicate significant effects.

	Treatment			**Plant population**			**Treatment * Plant population**		
	df	F	P	df	F	P	df	F	P
2012									
Total abundance	5	0.432	0.852	1	10.950	**0.001**	5	2.026	0.083
Herbivore abundance	5	0.403	0.846	1	9.387	**0.003**	5	1.691	0.146
Carnivore abundance	5	0.696	0.628	1	5.826	**0.018**	5	2.305	0.052
Total species richness	5	0.432	0.832	1	0.336	0.563	5	2.021	0.084
Herbivore species richness	5	0.741	0.595	1	1.233	0.270	5	2.184	0.063
Carnivore species richness	5	0.703	0.623	1	0.142	0.707	5	1.222	0.306
2013									
Total abundance	2	0.486	0.619	1	0.052	0.821	2	0.028	0.973
Herbivore abundance	2	0.461	0.634	1	0.568	0.455	2	0.243	0.786
Carnivore abundance	2	0.471	0.628	1	0.002	0.968	2	0.250	0.780
Total species richness	2	0.517	0.600	1	0.002	0.961	2	0.351	0.706
Herbivore species richness	2	0.582	0.564	1	0.207	0.651	2	0.435	0.650
Carnivore species richness	2	0.170	0.844	1	0.014	0.907	2	0.152	0.860

Supplementary Table 3. ANOVAs of plot scores (PCA) of whole arthropod community during different parts of the season (2013). Unconstrained ordination (PCA) with week number as covariate of all field plots of WIN or KIM plants receiving early season herbivore induction resulted in scores of arthropod community ordination (all species included except *B. brassicae* and *P. xylostella*) per individual plot on the first, second and third ordination axis. Factors 'Treatment' (order of herbivore Induction*Plant population interaction combinations: WIN A&C, WIN A-C, WIN C-A, KIM A&C, KIM A-C, KIM C-A), and monitored rounds (1: week 21-25; 2: week 25-33) and their interactions were tested with ANOVA for effects on plot scores on the first three axes. Numbers in **bold** indicate significant effects. When main effect 'Treatment' was significant, pair-wise comparisons between all herbivore induction*plant population combinations were performed using LSD post hoc test; only significant pairs are given.

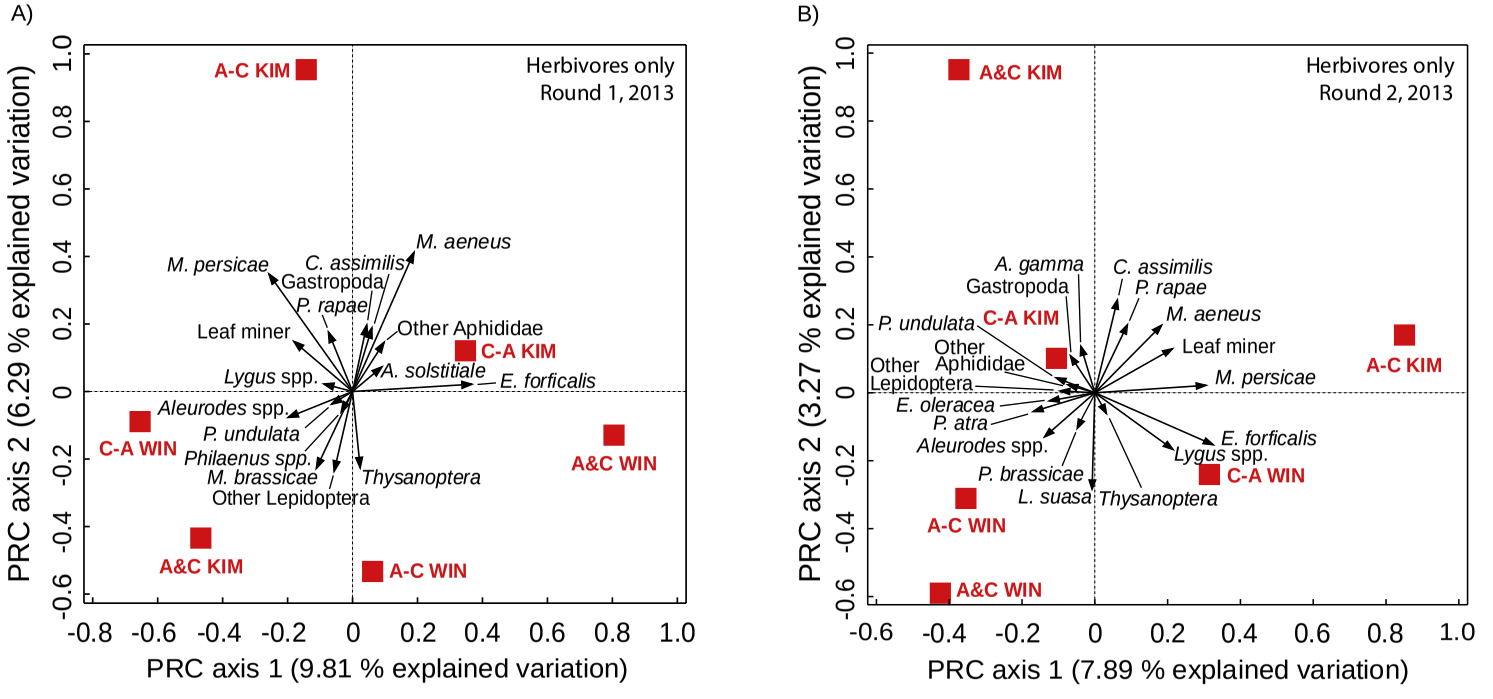
Monitored rounds	PCA axis	Factors tested (F_{df}, P)			Significant pairwise difference between order of herbivore arrival * plant population-combinations (LSD post-hoc results)
		Treatment (Induction * Plant pop.)	**Round**	**Treatment * Round**	
R1 + R2	1	F_5=1.90; 0.104	F_1<0.01; 0.955	F_5=1.64; 0.159	
R1 + R2	2	F_5=1.05; 0.396	F_1<0.01; 0.984	**F_5=2.45; 0.041**	
R1 + R2	3	**F_5=2.61; 0.031**	F_1=0.23; 0.634	F_5=0.57; 0.720	A&C WIN vs A-C KIM; A&C WIN vs C-A WIN; A-C KIM vs A-C WIN; A-C KIM vs C-A KIM
R1	1	**F_5=2.70; 0.034**	-	-	A&C KIM vs A&C WIN; A&C WIN vs C-A WIN; C-A KIM vs C-A WIN
R1	2	**F_5=2.94; 0.024**	-	-	A&C KIM vs A-C KIM; A&C WIN vs A-C KIM; A-C KIM vs A-C WIN; A-C KIM vs C-A KIM
R1	3	F_5=0.30; 0.907	-	-	
R2	1	F_5=1.87; 0.121	-	-	
R2	2	F_5=1.29; 0.288	-	-	
R2	3	F_5=1.14; 0.353	-	-	

Supplementary Table 4. ANOVAs of plot scores (PCA) of herbivore community during different parts of the season (2013). Unconstrained ordination (PCA) with week number as covariate of all field plots of WIN or KIM plants receiving early season herbivore induction resulted in scores of herbivore-only community ordination (all herbivore species included except *B. brassicae* and *P. xylostella*) per individual plot on the first, second and third ordination axis. Factors 'Treatment' (order of herbivore Induction*Plant population interaction combinations: WIN A&C, WIN A-C, WIN C-A, KIM A&C, KIM A-C, KIM C-A), and monitored rounds (1: week 21-25; 2: week 25-33) and their interactions were tested with ANOVA for effects on plot scores on the first three axes. Numbers in **bold** indicate significant effects. When main effect 'Treatment' was significant, pair-wise comparisons between all herbivore induction*plant population combinations were performed using LSD post hoc test; only significant pairs are given.

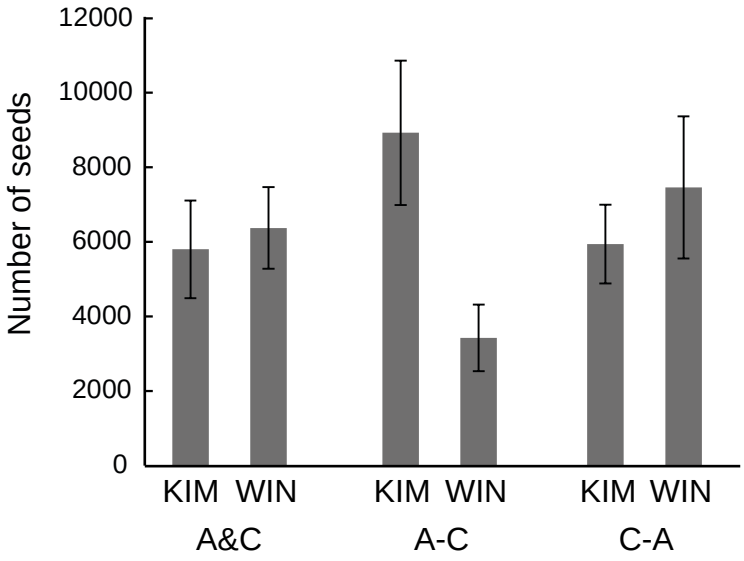
Monitored rounds	PCA axis	Factors tested (F_{df}, P)			Significant pairwise difference between order of herbivore arrival * plant population-combinations (LSD post-hoc results)
		Treatment (Induction * Plant pop.)	**Round**	**Treatment* Round**	
R1 + R2	1	F_5=1.85; 0.112	F_1=0.01; 0.941	F_5=2.04; 0.082	
R1 + R2	2	**F_5=2.53; 0.036**	F_1=0.24; 0.624	F_5=0.44; 0.817	A&C WIN vs A-C KIM; A-C KIM vs A-C WIN; A-C KIM vs C-A KIM
R1 + R2	3	F_5=1.49; 0.201	F_1=0.13; 0.716	F_5=1.38; 0.242	
R1	1	**F_5=2.72; 0.033**	-	-	A&C KIM vs A&C WIN; A&C WIN vs C-A WIN; C-A KIM vs C-A WIN
R1	2	**F_5=2.59; 0.041**	-	-	A&C KIM vs A-C KIM; A&C WIN vs A-C KIM; A-C KIM vs A-C WIN; A-C KIM vs C-A KIM
R1	3	F_5=0.48; 0.791	-	-	
R2	1	F_5=1.61; 0.179	-	-	
R2	2	F_5=0.47; 0.798	-	-	
R2	3	F_5=1.70; 0.157	-	-	

Supplementary Table 5. ANOVAs of plot scores (PCA) of arthropod community found on different plant parts during different parts of the season (2013). Unconstrained ordination (PCA) with week number as covariate of all field plots of WIN or KIM plants receiving early season herbivore induction resulted in scores of the whole arthropod community ordination found on the whole plant, reproductive part (flowers) or vegetative part (leaves) of the plant (all arthropod species included except *B. brassicae* and *P. xylostella*) per individual plot on the first, second and third ordination axis. Factors 'Treatment' (order of herbivore Induction*Plant population interaction combinations: WIN A&C, WIN A-C, WIN C-A, KIM A&C, KIM A-C, KIM C-A), and monitored rounds (1: week 21-25; 2: week 25-33) and their interactions were tested with ANOVA for effects on plot scores on the first three axes. Numbers in **bold** indicate significant effects. When main effect 'Treatment' was significant, pair-wise comparisons between all herbivore induction*plant population combinations were performed using LSD post hoc test; only significant pairs are given.

Plant part-associated community	PCA axis	Factors tested (F_{df}, P)			Significant pairwise difference between order of herbivore arrival * plant population-combinations (LSD post-hoc results)
		Treatment (Induction * Plant pop.)	**Round**	**Treatment * Round**	
Flowers+ Leaves	1	F_5=1.91; 0.102	F_1<0.01; 0.964	F_5=1.86; 0.111	
Flowers+ Leaves	2	F_5=1.65; 0.156	F_1<0.01; 0.990	F_5=2.01; 0.086	
Flowers+ Leaves	3	F_5=2.25; 0.058	F_1=0.30; 0.584	F_5=0.67; 0.644	
Flowers	1	F_5=2.02; 0.085	F_1=0.03; 0.855	F_5=2.11; 0.072	
Flowers	2	**F_5=2.78; 0.023**	F_1=0.01; 0.917	**F_5=2.56; 0.033**	A&C KIM vs A-C KIM; A&C WIN vs A-C KIM; A-C KIM vs A-C WIN
Flowers	3	F_5=0.60; 0.699	F_1=0.08; 0.782	F_5=1.15; 0.342	
Leaves	1	F_5=1.46; 0.212	F_1<0.01; 0.991	F_5=1.74; 0.134	
Leaves	2	F_5=1.71; 0.143	F_1=0.31; 0.578	F_5=0.82; 0.539	
Leaves	3	F_5=1.77; 0.129	F_1<0.01; 0.944	F_5=0.54; 0.744	



Supplementary Figure 1. Partial Redundancy Analysis showing the ordination of herbivore species in the community and the order of early herbivore arrival*plant population interactions, early and mid- season 2013. Shown are the first two ordination axes of the herbivore community in a) round 1 (week 21-25) and b) round 2 (week 25-33). Squares indicating factors (order of early season herbivore arrival * plant population interaction), and vector arrows indicating species ordination. All 20 herbivore species, but excluding *B. brassicae* and *P. xylostella*, are shown (some species only occurred during one of the rounds). Cumulative explained variation over the first four ordination axes: 18.85% (round 1) and 13.93% (round 2).



Supplementary Figure 2. Number of seeds per order of herbivore arrival treatment and plant population. Average number of seeds per plant (±SE) are shown for: Early season herbivory treatments: A&C: aphid *B. brassicae* & caterpillar *P. xylostella* simultaneously; C-A: caterpillars, 6 days later followed by aphids; A-C: aphids, 6 days later followed by caterpillars; and Plant population: KIM: Kimmeridge; WIN: Winspit.

Chapter 5

Plant-mediated interactions between two herbivores differentially affect a subsequently arriving third herbivore in populations of wild cabbage

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Abstract

Plants are part of biodiverse communities, and frequently suffer from attack by multiple herbivorous insects. Plant responses to these herbivores are specific for insect feeding guilds: aphids and caterpillars induce different plant phenotypes. Moreover, plants respond differentially to single or dual herbivory, which may cascade into a chain of interactions in terms of resistance to other community members. Whether differential responses to single or dual herbivory have consequences for plant resistance to yet a third herbivore is unknown.

We assessed the effects of single or dual herbivory by *Brevicoryne brassicae* aphids and/or *Plutella xylostella* caterpillars on resistance of plants from three natural populations of wild cabbage to feeding by caterpillars of *Mamestra brassicae*. We measured plant gene expression and phytohormone levels to illustrate mechanisms involved in induced responses.

Performance of both *B. brassicae* and *P. xylostella* was reduced when feeding simultaneously with the other herbivore, compared to feeding alone. Gene expression and phytohormone levels in plants exposed to dual herbivory were different from those found in plants exposed to herbivory by either insect alone. Plants previously induced by both *P. xylostella* and *B. brassicae* negatively affected growth of the subsequently arriving *M. brassicae*. Furthermore, induced responses varied between wild cabbage populations.

Feeding by multiple herbivores differentially activates plant defences, which has plant-mediated negative consequences for a subsequently arriving herbivore. Plant population-specific responses suggest that plant populations adapt to the specific communities of insect herbivores. Our study contributes to the understanding of plant defence plasticity in response to multiple insect attack.

Keywords

Brassica oleracea, *Brevicoryne brassicae*, *Mamestra brassicae*, multiple herbivory, phytohormones, plant defence, *Plutella xylostella*

Introduction

Throughout the growing season, plants suffer from attack by multiple herbivorous insects. To reduce insect attack, plants protect themselves with constitutive defences like thick cell walls, a waxy epidermal cuticle, or toxins (Schoonhoven *et al.* 2005). Furthermore, plants show defence responses induced by herbivores, for example by producing compounds that deter or repel the attackers (Schoonhoven *et al.* 2005), which may also affect subsequently feeding herbivores (Kessler & Halitschke 2007). Depending on the feeding guild of the attacking insect, changes in phytohormone production, gene transcription and protein production can occur, which lead to a different regulation of plant defences (Heidel & Baldwin 2004; de Vos *et al.* 2005; Bidart-Bouzat & Kliebenstein 2011; Koo *et al.* 2013) and, thus, to expression of a herbivore species-specific induced plant phenotype.

Phytohormonal signalling networks underlying herbivore-induced defence responses involve, amongst others, two major phytohormones: jasmonic acid (JA) and salicylic acid (SA) (Pieterse *et al.* 2012). In general, JA-mediated signalling underlies plant defence responses against chewing herbivores (Stam *et al.* 2014). Lipoxygenases (LOXs) are important enzymes involved in JA biosynthesis (Turner *et al.* 2002). In cabbage, insect herbivory by *Pieris rapae*, *Pieris brassicae* or *Mamestra brassicae* caterpillars induced high transcript levels of *BoLOX* (Broekgaarden *et al.* 2007; Zheng *et al.* 2007). The importance of JA in defence responses against insects was also shown in other plant species such as tomato (*Solanum lycopersicum*) (Thaler *et al.* 2002a), milkweed (*Asclepsias syriaca*) (Ali & Agrawal 2014) and tobacco (*Nicotiana attenuata*) (reviewed by Kessler & Baldwin 2004; Wang & Wu 2013). Phloem feeders induce SA-regulated defences (de Vos *et al.* 2005). Regulation of SA-mediated defences involves the expression of pathogenesis-related (*PR*) genes. For example, Kuśnierczyk *et al*. (2008) investigated transcriptomic changes of *Arabidopsis* in response to feeding by *Brevicoryne brassicae* aphids and found that the expression of PR genes was significantly induced.

Plants regulate induced defences against attacking herbivores through crosstalk between JA and SA signalling pathways (Pieterse *et al.* 2009). For example, in *Nicotiana attenuata* plants crosstalk between the JA and SA signalling pathways resulted in optimization of defence responses (Rayapuram & Baldwin 2007). However, insect herbivores can also interfere with JA- and SA-induced defences, which can affect the outcome of interactions between plants and multiple attackers (Voelckel & Baldwin 2004; Rodriguez-Saona *et al.* 2010; Mathur *et al.* 2013). Through these indirect plant-mediated interactions, competition between attacking herbivores is commonly found in nature in which induced plant responses to a first herbivore may affect the resistance of plants to subsequent herbivores (Denno *et al.* 1995; Kaplan & Denno 2007). Asymmetric interactions between herbivores seem to be the rule rather than the exception (Kaplan & Denno 2007), which could lead to positive (i.e. facilitation) or negative (i.e. antagonism) effects on the performance

or preference of the competing herbivore species (Kessler & Halitschke 2007; Viswanathan *et al.* 2007; Erb *et al.* 2011; Soler *et al.* 2012a; Ali & Agrawal 2014; Li *et al.* 2014). For instance, monarch caterpillars developed faster on milkweed plants previously infested by oleander aphids, whereas the aphids developed more slowly on milkweed plants previously infested by caterpillars, which might have been JA-mediated (Ali & Agrawal 2014).

Next to the effect of one feeding herbivore on induced defences against a subsequently arriving herbivore, plant responses to dual stresses can have further ecological consequences for interactions with other community members (Utsumi *et al.* 2010). However, this is not yet studied in great detail. Recently, it has been shown that the specialist caterpillar *Plutella xylostella* gained more weight when feeding on plants previously attacked by both *P. xylostella* and *Spodoptera litura* caterpillars compared with plants previously infested by only *P. xylostella*. In contrast, *S. litura* was negatively affected when feeding on plants previously attacked by both *P. xylostella* and *S. litura,* compared with plants previously infested by only *P. xylostella* (Mathur *et al.* 2013). The effect of dual herbivory on a third herbivore suggests that interspecific competition between multiple herbivores can have consequences for the composition of whole arthropod communities assembling on the induced plant. Consequently, interactions between plants and co-occurring insects likely play important roles in natural ecosystems (van Zandt & Agrawal 2004b; Ali & Agrawal 2014; Poelman & Kessler 2016). Effects of early-season herbivores on arthropod community development have indeed been shown several times (van Zandt & Agrawal 2004b; Viswanathan *et al.* 2007; Poelman *et al.* 2010).

Studies on temporal dynamics of plant defences in response to herbivory indicate that plants may remain induced by herbivory up to several days (Voelckel & Baldwin 2004; Underwood 2012; Mathur *et al.* 2013). However, induced defences were investigated after short periods of herbivory, after which the herbivore had been removed. Under natural conditions, insects likely arrive at different times on a plant, and more research is needed to study underlying molecular mechanisms in plants induced during different durations of continuous feeding and consequences for subsequent herbivores. In addition, closely related plant species differ in responses to herbivore attack which may affect interactions between two or more insect species associated with the plant (Johnson & Agrawal 2005; Agrawal *et al*. 2014). Even within plant species, populations may differ in the amount of secondary metabolites they produce (Gols *et al.* 2008b), which has consequences for the insect communities on those populations (Newton *et al*. 2009b; Poelman *et al*. 2009; Li *et al*. 2014).

In the present study, we used wild *B. oleracea* plants that occur in natural populations along the coast of Dorset, UK. These plants show natural variation in the amount of constitutive and inducible secondary metabolites that act as defence

compounds against herbivorous insects (Gols *et al*. 2008; Newton *et al*. 2009a). Under natural conditions, these plants are attacked by an array of herbivorous insects, amongst others the specialist aphid *Brevicoryne brassicae*, the specialist caterpillar *P. xylostella* and the generalist caterpillar *Mamestra brassicae* (R. Gols, pers. comm.; Moyes *et al.* 2000). Also in the Netherlands, these insects naturally occur throughout the growing season on *B. oleracea* cultivars (Poelman *et al.* 2009) and wild *B. oleracea* plant populations (J. M. Stam; unpublished data). The aphid *B. brassicae* and caterpillars of *P. xylostella* commonly colonize *B. oleracea* plants early in the season, while the caterpillars of *M. brassicae* arrive later in the season. Thus, *M. brassicae* caterpillars may encounter plants that have been previously attacked by *P. xylostella*, *B. brassicae* or both of these herbivores. We investigated whether herbivores from different feeding guilds, namely aphids (*Brevicoryne brassicae*) or caterpillars (*P. xylostella*) feeding alone or simultaneously indeed affected performance of the competing herbivore, and whether this was reflected in expression changes of marker genes involved in defence responses, i.e. *PR-1* and *LOX* (regulated by the phytohormones SA and JA respectively). We quantified JA and SA levels in wild cabbage plants to assess differences in phytohormone levels in response to dual versus single herbivory. By using plants from different wild cabbage populations from the same area, we studied whether plant defence responses are variable across these plant populations. As a next step in the study of multiple interacting herbivores, we studied whether changes in plant resistance induced by the first two herbivores have consequences for the performance of a subsequently arriving generalist herbivore (*M. brassicae*). We discuss the ecological consequences of plant defence to multi-herbivory.

Materials and methods

Plants and growth conditions

Seeds of wild cabbage *Brassica oleracea* L. (Brassicaceae), from three populations in Dorset, i.e. Kimmeridge (50°36'N, 2°07'W), Old Harry (50°38'N, 1°55'W) and Winspit (50°35'N, 2°02'W) (Gols *et al.* 2008b) were sown in potting soil (Lentse potgrond, Lent, The Netherlands). One week later, seedlings were transferred to individual pots (1.54 L) containing similar soil. Plants were cultivated in a glasshouse under a 16L : 8D cycle [500 µmol photons m^{-2} s^{-1}] light intensity at 22 ± 3 °C and 50-70 % relative humidity. Lighting from high-pressure mercury lamps was used in the glasshouse to supplement periods of low natural light. Plants were watered every other day. When plants were four weeks old, they were used for experiments.

Insects

The specialist diamondback moth *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), the specialist cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) and the generalist cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) were obtained from stock cultures maintained at the Laboratory of Entomology, Wageningen University. All insects were reared on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* cv Cyrus) in a climate room (21±1 °C, 50-70 % relative humidity, 16L : 8D cycle).

Experimental set-up

The experiments were performed in three different rounds (November 2012, January/February 2013 and March/April 2013) to obtain three biological replicates. At time points 3, 7 or 14 d after insect infestation, performance of the insects were assessed, and plant tissue for molecular defence analyses was collected. For each time point, a different set of plants was used to exclude effects of sampling tissue for molecular analyses on insect performance. Therefore, a total of 432 plants (4 plants x 4 treatments x 3 time points x 3 *B. oleracea* populations x 3 rounds) was used over the entire study.

Plutella xylostella and *Brevicoryne brassicae* performance

At each round, 48 plants per *B. oleracea* population were infested with three second-instar (L2) *P. xylostella* caterpillars, or with five adult *B. brassicae* aphids, or simultaneously infested with three L2 *P. xylostella* caterpillars plus five adult *B. brassicae* aphids. Each insect was carefully placed with a small brush on the third fully expanded leaf. Clean uninfested plants were used as control. Immediately after placing the insects on a leaf, they were caged for 24 h by using clip cages; upon removal of the clip cages the insects were allowed to move and feed freely on the plant. Empty clip cages were used for 24 h on leaves of control plants. Individual (infested and control) plants were covered with a gauze net supported by four wooden sticks to prevent insects from escaping.

For insect performance, individual plants were considered as the unit of biological replication. At time points 3, 7 or 14 d after infestation *P. xylostella* caterpillars or pupae were collected per plant and individually weighed (analytical balance: Mettler Toledo ML54/01, accuracy = 0.1 mg), and number of *B. brassicae* aphids per plant was recorded.

Molecular plant defence analyses

For gene expression and phytohormone analysis, one biological replicate consisted of eight leaf discs punched with a cork-borer (diameter = 2.1 cm) and pooled from

four different plants. Plant material was collected after 3, 7 or 14 d from the leaf on which the clip cage had been fixed during the first 24 h. Because after the first 24 h the insects could freely move around on the plant, the transcription levels of genes measured were affected by both initial local induction as well as subsequent systemic feeding by the herbivores. Leaf discs were flash-frozen in liquid nitrogen and stored at -80 °C prior to analysis.

Quantitative real-time PCR

Total RNA was isolated from finely ground and homogenized leaf material with the RNeasy Plant Mini Kit (Qiagen). RNA was treated with DNaseI (Invitrogen) following the manufacturer's instructions. Subsequently cDNA was synthesised from RNA (adjusted to 1 µg/µl) using iScript cDNA synthesis Kit (Bio-Rad). Quantitative RT-PCR analysis was performed in a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Each reaction was performed in a total volume of 25 µl containing 12.5 µl SYBR Green Supermix (Bio-Rad), 5 µl cDNA and 1 µl of 10 µM forward and reverse gene specific primer pair. For each reaction, two technical replicates were performed and average values were used in the analyses. The following PCR program including a melting curve analysis was used for all PCR reactions: 3 min 95 °C, followed by 40 cycles of 15 sec 95 °C, and 45 sec 59 °C. Relative expression of a pathogenesis-related protein (*BoPR-1*) and lipoxygenase (*BoLOX*) were calculated by using the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen 2001) with the housekeeping gene *glyceraldehyde-3-phosphate dehydrogenase* (*GAPDH*) (Broekgaarden *et al.* 2008) as internal standard.

Primer sequences (Table 1) were based on genes of *Brassica oleracea var. gemmifera*, namely *BoLOX* (GenBank accession EF123056), *BoPR-1* (GenBank accession EF423806) and the reference gene *BoGAPDH* (GenBank accession EF123055).

Table 1. Specific primer sequences used for quantitative RT-PCR analyses.

Gene name	Forward primer	Reverse primer
BoGAPDH	AGAGCCGCTTCCTTCAACATCATT	TGGGCACACGGAAGGACATACC
BoLOX	AAGGCATCGAGCTTCCCAA	TTGCTTTTCAACGGCCACTC
BoPR-1	GTCAACGAGAAGGCTAACTATAACTACG	TTACACCTTGCTTTGCCACATCC

Phytohormone quantification

JA and SA phytohormone levels were quantified by gas chromatography – mass spectrometry as described by Schulze *et al.* (2006). Plant material (250 mg) was finely ground and frozen in liquid nitrogen. For quantification, [9,10-$^{2}H_2$]-9,10-dihydro-JA (250 ng) and [3,4,5,6-$^{2}H_4$]-SA (500 ng) were added as internal standards. JA levels were quantified by analysing samples on a Finnigan ITQ 900 (Thermo Scientific, Dreiech, Germany) device equipped with an Rtx-200MS column (30 m, 0.25 mm,

0.25 mm; Resteck, Bad Homburg, Germany). Helium (1.5 mL min^{-1}) served as the carrier gas. Mass spectral analysis was carried out in chemical ionization negative (NCI) mode using methane as reagent gas (2.0 mL min^{-1}). Products were eluted under programmed conditions: 100 °C, increase (10 °C min^{-1}) to 210 °C, increase (1 °C min^{-1}) to 227 °C, hold 1 min, increase (40 °C min^{-1}) to 290 °C, hold 2 min. The GC injector (split ratio 1:10), transfer line and ion source were set at 280, 300 and 200 °C, respectively.

SA levels were quantified by analysing samples on a Finnigan Trace MS quadrupole mass spectrometer (Thermo electron) according to Schulze *et al.* (2006).

Mamestra brassicae performance

At time points 4, 8 and 15 d after infestation, all 48 plants per population were sampled to investigate the effects of dual herbivory on a subsequently arriving herbivore, i.e. *M. brassicae* caterpillars. From each plant three leaves were excised: the leaf on which the clip cage had been fixed for the first 24 h and sampled for gene expression and phytohormone analyses, as well as two additional leaves which were fully unfolded and also damaged by insect feeding.

We used excised leaves to exclude that *M. brassicae* itself would induce systemic plant responses in addition to the induced plant phenotypes derived from single or dual herbivory and we thus aimed to arrest the plant phenotype induced by treatment with *B. brassicae* and/or *P. xylostella* feeding. We selected the leaf on which initial herbivores had been inoculated to link the measurements of gene expression and phytohormones directly to *M. brassicae* performance and added two leaves on which the initial herbivores had been feeding to provide ad libitum food to the *M. brassicae* caterpillars. These leaves, together representing one biological replicate, were placed in a small vial with tap water and sealed with a cotton plug. All insects were removed when the leaves were excised. Vials containing the leaves were placed in a plastic container (12 x 18 x 7 cm, L x W x H) covered with a transparent lid with 12 small holes. In each container 10 neonate (L1) *M. brassicae* caterpillars were carefully placed on the leaves with a small brush and allowed to feed for 6 d. Although after 6 d the leaves looked still healthy and remained turgid, we cannot exclude that leaf quality may have gradually reduced over the course of the experiment. Containers were placed in a glasshouse (22 ± 3 °C, 50-70 % relative humidity, 16L : 8D cycle). After 6 d of feeding, caterpillars were individually weighed on an analytical balance (Mettler Toledo ML54/01, accuracy = 0.1 mg). Mortality was calculated as the initial number of larvae placed on the leaves minus the number of larvae that were still alive at the moment of weighing.

Statistical analysis

The effects of herbivore treatments, time points, experimental rounds and plant populations on

(I) *B. brassicae* numbers, gene expression and phytohormone levels were analysed using a Generalized linear model (forward accumulated analysis of deviance) with Poisson distribution and log link function. Time point was included as covariate, while treatment, plant population and round were included as fixed factors. An estimated dispersion parameter was included to account for residual variance. When interactions between factors were not significant, only main treatment effects are presented.

(II) *P. xylostella* and *M. brassicae* caterpillar and pupal weights were analysed using a Generalized linear mixed model (sequentially adding terms to fixed model) with normal distribution and identity link function. The model included a random effect of individual plant identity and an estimated dispersion parameter to account for residual variance. When terms were not significant they were subsequently removed from the statistical model. In cases where *P. xylostella* caterpillar weight was lower than the accuracy of the balance (n = 31), they were entered with the lowest measurable weight (0.1 mg) in the analysis. Interaction terms between treatments, time points, experimental rounds and plant populations could not be computed for *M. brassicae* data because of an insufficient number of degrees of freedom.

(III) *M. brassicae* mortality and the fraction of *P. xylostella* pupae relative to the total of all *P. xylostella* life stages that were found per plant at each of the time points, were analysed using a Generalized linear model (forward accumulated analysis of deviance) with binomial distribution and logit link function. Binomial totals were always 10 *M. brassicae* larvae or were the totals of all *P. xylostella* life stages found per plant. An estimated dispersion parameter was included to account for residual variance.

Post-hoc tests for differences between levels of the fixed factors were analysed with a t-test for pairwise differences of the means for *B. brassicae* numbers and *M. brassicae* mortality. Post-hoc comparisons for *P. xylostella* and *M. brassicae* weights, gene expression and phytohormone levels were analysed with an LSD test. All statistical analyses were conducted in GenStat software Version 16.2 (VSN International, Hemel Hempstead, UK).

A correlation analysis was used to test for a relationship between JA and SA level, JA level and *LOX* expression, SA level and *PR-1* expression and *LOX* and *PR-1* expression in plants from each population and treatment.

Excluded from analysis were all samples (n = 38 plants over the entire study) that had unintended *B. brassicae* infestation.

Results

Performance of *B. brassicae* aphids and *P. xylostella* caterpillars

The number of *B. brassicae* that accumulated per plant was 42 % lower when *B. brassicae* aphids were feeding simultaneously with *P. xylostella* on the same plant, compared to *B. brassicae* aphids feeding alone on Kimmeridge plants (Table 2A, Figure 1). Aphid numbers were not affected by plant population and increased significantly with time. The experimental rounds significantly influenced *B. brassicae* numbers (Table 2A).

Simultaneous feeding by *B. brassicae* affected *P. xylostella* caterpillar weight negatively, depending on the time point and plant population (Table 2B). Thus, both *B. brassicae* and *P. xylostella* performance were negatively affected by simultaneous feeding by the reciprocal herbivore, and influenced by plant population or time of infestation. At 3 d after induction, there was no difference in caterpillar weights between the treatments, whereas at 7 d after induction, caterpillar weight was 16 % lower when *P. xylostella* caterpillars were feeding simultaneously with *B. brassicae* aphids on the same plant, compared to *P. xylostella* caterpillars feeding

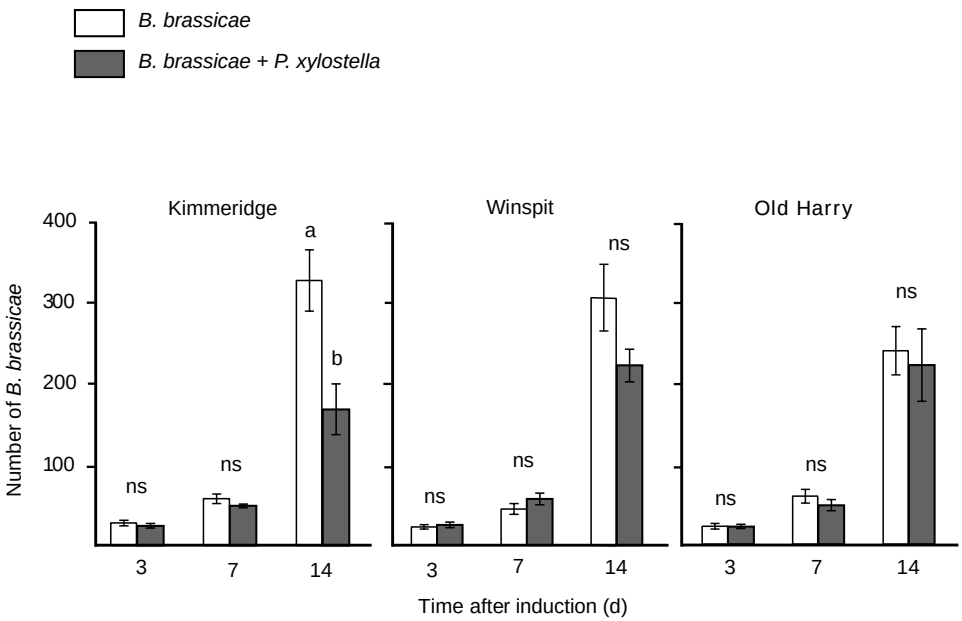

Figure 1. *Brevicoryne brassicae* performance. Mean number of *B. brassicae* (± SE) on plants of three wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 3, 7 or 14 days after single *B. brassicae* or simultaneous *P. xylostella* and *B. brassicae* infestation. Bars marked with different letters are significantly different (GLM, $P < 0.05$); ns indicates no significant difference between groups.

alone (Figure 2). Plant population affected *P. xylostella* caterpillar weights; highest weight at 7 d after induction was reached on Old Harry plants, lowest weight was reached on Winspit plants (Figure 2). Experimental rounds affected *P. xylostella* caterpillar weight. Pupal weights were neither affected by the presence or absence of *B. brassicae* on the plant (Table 2A), nor by plant population, experimental round or time point. At 14 d, the majority of the *P. xylostella* caterpillars had pupated and a small fraction of the pupae had eclosed. Likewise, *P. xylostella* development time until pupation, measured as the fraction of pupation per time point, was not affected by simultaneous feeding by *B. brassicae* on the same plant (Table 2A). Neither differences between plant populations, nor between experimental rounds were significant; the fraction of pupation increased over time.

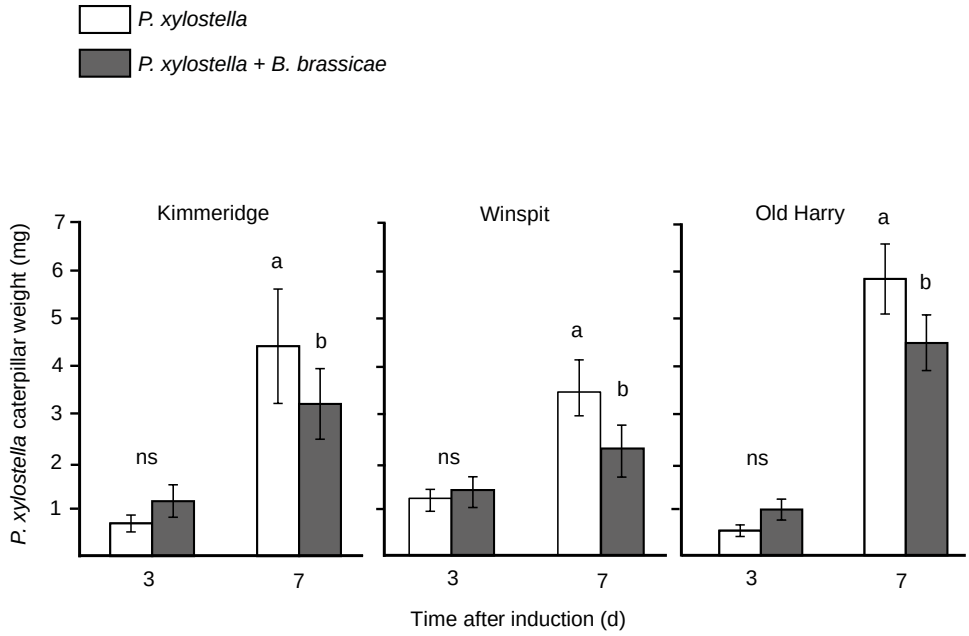

Figure 2. *Plutella xylostella* performance. Mean weight (milligrams) of *P. xylostella* caterpillars (± SE) on plants of three wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 3 or 7 days after single *P. xylostella* or simultaneous *P. xylostella* and *B. brassicae* infestation. Data for 14 days after induction are not shown here, because only few *P. xylostella* in the caterpillar stage remained at that time point; the rest pupated. Bars marked with different letters are significantly different (GLMM, $P < 0.05$); ns indicates no significant difference between groups within a time point.

Transcriptional analyses

Expression of the SA-responsive marker gene *PR-1* was significantly affected by treatment, plant population and time point (Table 3A) as well as their interaction

Table 2. Statistical tests on performance variables of insects feeding on plants from three wild *B. oleracea* plant populations, which were either undamaged or induced by P*lutella xylostella* caterpillars, *Brevicoryne brassicae* aphids, or both. (a) Generalized Linear Model deviance table for *Brevicoryne brassicae* numbers, fraction of *Plutella xylostella* pupae and *Mamestra brassicae* caterpillar mortality. (b) Generalized Linear Mixed Model Wald table for *Plutella xylostella* caterpillar weights, *Plutella xylostella* pupal weights, *Mamestra brassicae* caterpillar weights and *Mamestra brassicae* caterpillar weights on control plants only. Bold numbers indicate significant effects of the factor on insect performance (α = 0.05). [a] ns: indicates a non-significant factor that was not included in the model.

(A)	Generalized Linear Model deviance table – Factors												Total Model	
	Treatment			Plant population			Time point			Round				
	d.f.	deviance	P	d.f.	deviance	P	d.f.	deviance	P	d.f.	deviance	P	d.f.	deviance
B. brassicae number	1	388.54	**<.001**			ns[a]	1	17811.26	**<.001**	2	1012.50	**<.001**	204	23749.81
P. xylostella pupal fraction			ns			ns	6	40.96	**<.001**			ns	120	217.37
M. brassicae caterpillar mortality			ns			ns	8	157.48	**<.001**			ns	391	959.05

(B)	Generalized Linear Mixed Model Wald table – Factors											
	Treatment			Plant population			Time point			Round		
	d.f.	F	P	d.f.	F	P	d.f.	F	P	d.f.	F	P
P. xylostella caterpillar weight	1	8.93	**0.003**	2	5.72	**0.004**	1	545.96	**<.001**	2	5.59	**0.004**
P. xylostella pupal weight	1	0.29	0.593	2	0.71	0.494	1	2.93	0.091	2	2.92	0.060
M. brassicae caterpillar weight	3	3.72	**0.012**	2	69.07	**<.001**	1	18.48	**<.001**	2	54.43	**<.001**
M. brassicae caterpillar weight control only				2	13.54	**<.001**	2	3.10	**0.050**	2	24.07	**<.001**

(Table 3B). Treatment, time point, plant population and round also had a significant effect on the expression of the JA-responsive marker gene *LOX* (Table 3A); however, there was no significant interaction between the factors treatment, plant population and time point (Table 3B).

Caterpillars feeding on Kimmeridge plants and simultaneous feeding by caterpillars and aphids on Old Harry plants significantly up-regulated *PR-1* expression 14 d after infestation (Figure 3). At this time point most of the *P. xylostella* caterpillars had pupated.

Significantly higher expression levels of *LOX* were found in all three cabbage populations upon aphid feeding alone and simultaneous aphid and caterpillar feeding compared to control plants and plants induced with caterpillars only (Figure 3).

In conclusion, after prolonged herbivory SA-mediated signalling is still upregulated and dual herbivory differentially affected JA-dependent *LOX* expression compared with plants infested by only *P. xylostella* caterpillars or control plants.

Phytohormonal analyses

To further investigate the effect of dual herbivore attack on plant defences, the levels of the phytohormones JA and SA were assessed. The level of JA was significantly affected by time point and experimental round (Table 3A). However, JA levels were similar among treatments for all time points and cabbage populations (Figure 4). There was a significant interaction between the effects of treatment, time point and plant population on SA level (Table 3B, GLM).

The level of SA was significantly higher in Kimmeridge plants induced by aphids only or by caterpillars only compared to plants simultaneously induced by both insects at 3 d (Figure 4). This indicates that in Kimmeridge plants aphids and caterpillars alone induce a different SA-mediated defence response compared to both insects feeding simultaneously.

Among the three plant populations, SA levels differed upon insect infestation. Aphid feeding induced significantly higher levels of SA in Winspit plants after 14 d compared to caterpillar-infested plants (Figure 4). In Kimmeridge plants, 14 d after caterpillar feeding, the SA level was significantly induced to higher levels than in plants simultaneously induced by both insects and in aphid-infested plants (Figure 4). This is a similar activation of the SA pathway (higher *PR-1* expression) as seen before in Kimmeridge plants (Figure 3). For aphid-infested Old Harry plants, SA level was significantly reduced compared to control and caterpillar-infested Old Harry plants 3 d after insect feeding (Figure 4).

In conclusion, dual herbivory by aphids and caterpillars resulted in a different phytohormonal response compared to phytohormonal responses induced by aphids or caterpillars alone. Furthermore, in response to herbivory SA levels were different across plant populations.

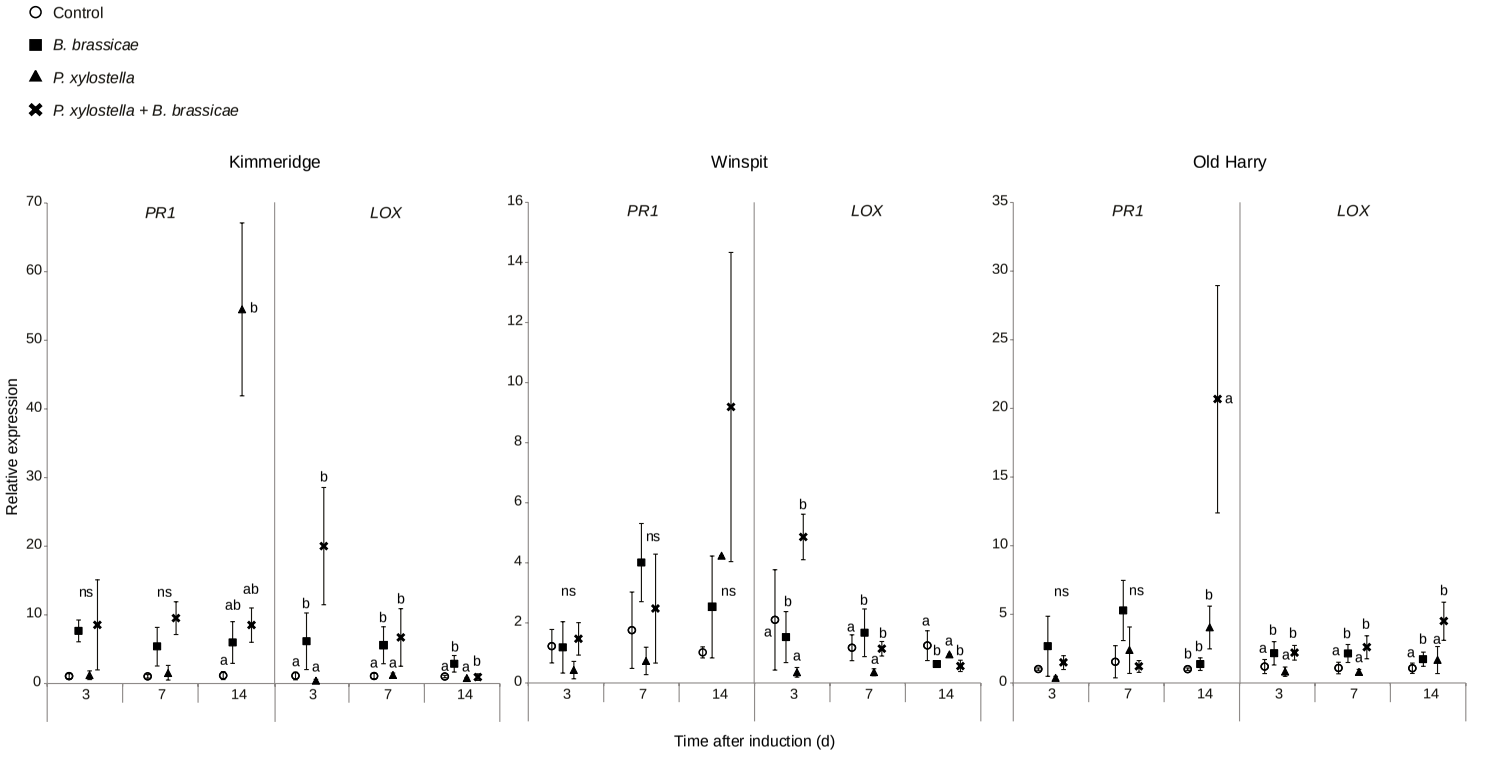

Figure 3. Gene expression in response to single or dual insect attack. *PR-1* and *LOX* expression in leaves of plants from wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 3, 7 or 14 days after single *P. xylostella*, single *B. brassicae*, simultaneous *P. xylostella* and *B. brassicae* infestation and without feeding (Control). Symbols represent means ± SE (n=3). Symbols marked with different letters are significantly different within a time point (GLM, $P < 0.05$); ns indicates no significant difference between groups within a time point.

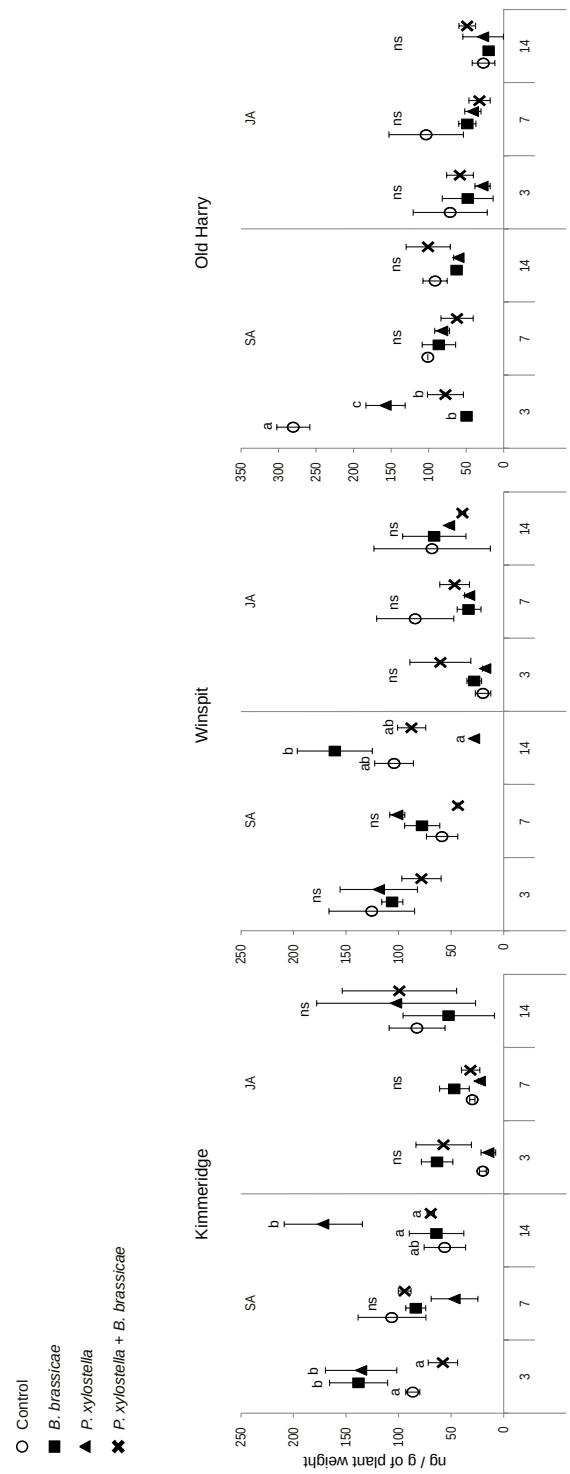

Figure 4. Phytohormone production in response to single or dual insect attack. Salicylic acid (SA) and jasmonic acid (JA) levels expressed as ng per g fresh weight in wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 3, 7 or 14 days after single *P. xylostella*, single *B. brassicae*, simultaneous *P. xylostella* and *B. brassicae* infestation and without feeding (Control). Symbols represent means ± SE (n=3). Symbols marked with different letters are significantly different within a time point (GLM, $P < 0.05$); ns indicates no significant difference between groups within a time point.

Table 3. Statistical tests on gene expression and hormone levels in plants from three wild *B. oleracea* populations, which were either undamaged or induced by *Plutella xylostella* caterpillars, *Brevicoryne brassicae* aphids, or both. Generalized Linear Model deviance table for effect of Time point, Treatment, Round and Population (a) and their interaction term (b). Non-significant interaction terms were not included in the model. Bold numbers indicate significant effects (α = 0.05).

(A)	**Generalized Linear Model deviance table – Factors**							
	Treatment (1)		Plant population (2)		Time point (3)		Round (4)	
	d.f. = 3		d.f. = 2		d.f. = 1		d.f. = 2	
	deviance	P	deviance	P	deviance	P	deviance	P
PR-1	139.755	**<.001**	198.65	**<.001**	168.699	**<.001**	10.684	0.144
LOX	82.38	**<.001**	45.01	**<.001**	64.98	**<.001**	72.97	**<.001**
SA	259.82	**0.025**	14.47	0.736	105.78	**0.044**	13.18	0.756
JA	92.02	0.163	24.89	0.481	101.75	**0.020**	401.66	**<.001**

(B)	**Generalized Linear Model deviance table - Interactions**													
	1 x 2		1 x 3		1 x 4		2 x 3		3 x 4		1 x 2 x 3		1 x 3 x 4	
	d.f. = 2		d.f. = 3		d.f. = 6		d.f. = 2		d.f. = 2		d.f. = 6		d.f. = 6	
	deviance	P	deviance	P	deviance	P	deviance	P	deviance	P	deviance	P	deviance	P
PR-1			133.71	**<.001**							46.902	**0.012**		
LOX	21.677	**0.005**					30.345	**<.001**	15.316	**0.001**			14.912	**0.027**
SA	395.60	**0.031**									373.12	**0.040**		
JA					355.50	**0.011**	173.03	**0.013**						

Performance of third subsequent herbivore *M. brassicae*

Plant resistance was altered by single or simultaneous feeding by the two herbivores *P. xylostella* and *B. brassicae* which negatively affected the third herbivore, *M. brassicae*, subsequently arriving on the same plant. *Mamestra brassicae* performance was 19 % lower on plants previously induced by both *P. xylostella* and *B. brassicae* compared to control plants without previous insect feeding (overall treatment effect; Table 2B; Figure 5). The performance of *M. brassicae* on undamaged plants or plants previously induced by either aphids or caterpillars did not differ from each other, indicating that only induction by the two herbivores together negatively affected the performance of *M. brassicae*.

Mamestra brassicae performance was affected by the length of time previous herbivores had spent feeding, as weight of *M. brassicae* caterpillars differed between the time points, mostly between 4 and 15 d; and 8 and 15 d after the start of previous herbivory. The three plant populations affected *M. brassicae* caterpillar weight differently, with lowest weight obtained on Winspit plants and highest weight on Kimmeridge plants. Also experimental round affected *M. brassicae* weight (Table 2B).

To verify whether the observed differences in *M. brassicae* weight were caused only by previous feeding by the inducing herbivores, or could also have been affected by differences in plant quality between the time points or plant populations, weight of *M. brassicae* caterpillars feeding on the control plants was analysed separately. *Mamestra brassicae* caterpillar weight was 20 % lower after feeding on control plants 15 d after onset of the experiment compared to *M. brassicae* weight feeding on control plants 4 and 8 d after onset of the experiment (Table 2B). On Winspit control plants, *M. brassicae* caterpillar weight was 47 % lower compared to control plants of the two other plant populations. Weight of *M. brassicae* was also affected by the experimental round when feeding from control plants.

Mortality of *M. brassicae* caterpillars was not affected by previous induction by aphids or caterpillars (Table 2A), and was neither affected by plant population nor by experimental round; only at the 14 d time point, mortality was higher than at either of the two other time points.

Discussion

In nature, plants are frequently under attack by multiple insect herbivores. Insects feeding on plants interact indirectly through plant-mediated effects in which initial insect attackers affect plant responses that influence subsequently feeding herbivores (Denno *et al.* 1995; Kaplan & Denno 2007). In addition, it is known that induction of plant defence responses differs between dual and single herbivore attack (Voelckel & Baldwin 2004). Importantly, the majority of herbivores will find

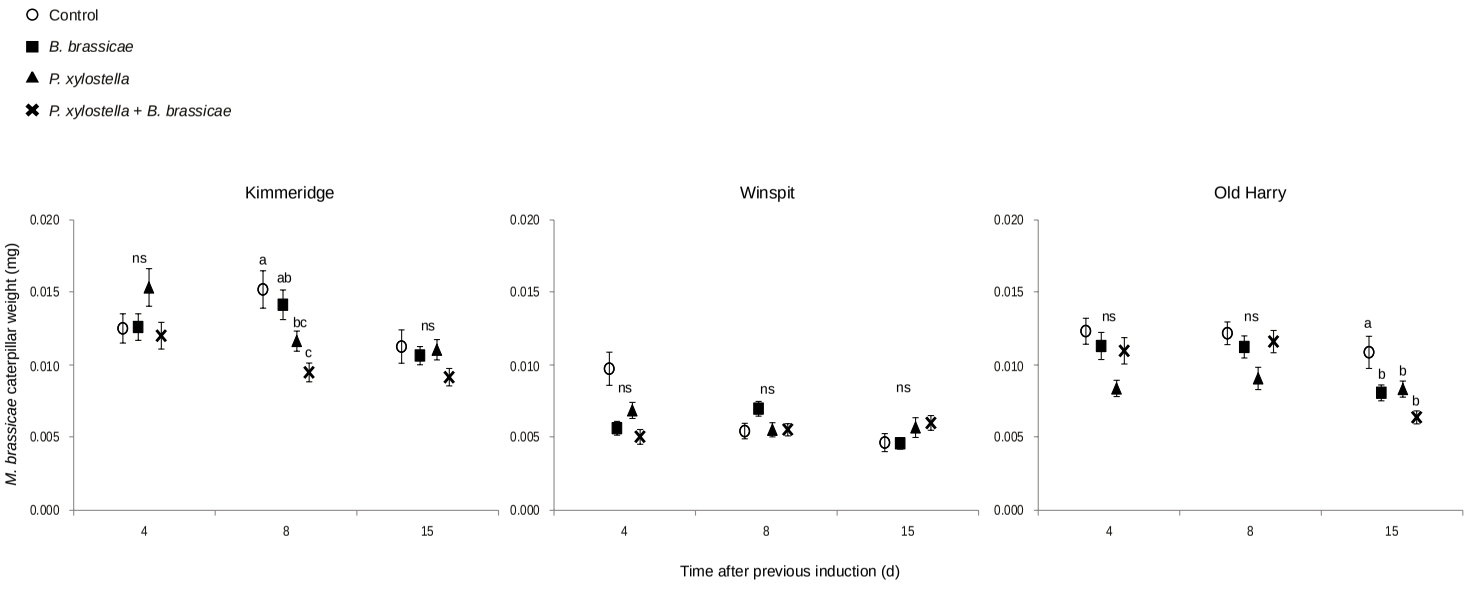

Figure 5. *Mamestra brassicae* performance. Mean weight (mg) of *M. brassicae* caterpillars (± SE) on plants of wild cabbage populations (Kimmeridge, Winspit and Old Harry) at 4, 8 or 15 days after previous infestation with single *B. brassicae*, single *P. xylostella* or simultaneous *P. xylostella* and *B. brassicae* and undamaged plants (Control). Symbols marked with different letters are significantly different (GLMM, $P < 0.05$); ns indicates no significant difference between groups within a time point.

themselves feeding on plants previously attacked by multiple insects, but little is known about the effect of multi-herbivore-induced plant phenotypes on resistance to subsequent attackers. We found that simultaneous feeding by *P. xylostella* and *B. brassicae* resulted in different plant defence-related gene expression and differences in plant hormone levels compared to single herbivory, and this had a negative effect on subsequently arriving *M. brassicae* caterpillars, depending on plant population and time point.

Here, the performance of both *P. xylostella* caterpillars and *B. brassicae* aphids was negatively affected by simultaneous feeding by the reciprocal herbivore. In contrast, in previous studies, positive reciprocal interactions between insects have been observed (Rieske & Raffa 1998; Poelman *et al.* 2008a; Soler *et al.* 2012a; Mathur *et al.* 2013; Agrawal *et al.* 2014; Li *et al.* 2014). Most of those studies concerned sequential insect infestation (i.e. one herbivore after the other). Here, we introduced the two initial herbivore species simultaneously which might explain the negative reciprocal effects on their performance that we recorded in this study. Sequential insect infestation causes a time lag between the induction by the first and a second attacker. Because plant defence signalling pathways are known to interact, a time lag could affect the interaction between defence signalling in a different way than when both attackers arrive at the same time (Erb *et al.* 2011; Karban 2011).

Not only the simultaneously attacking insects but also the subsequently arriving *M. brassicae* caterpillars were negatively affected by dual-herbivore-induced plant resistance. These findings suggest that plant responses to herbivores attacking alone affect an herbivore arriving later in a different way than simultaneously attacking herbivore species do (see also Kaplan & Denno 2007). Such trait-mediated interaction networks (Utsumi *et al.* 2010) imply that herbivores can have far-reaching consequences for not only the plant they feed on, but also for all later arriving insects. It has been previously reported that the generalist herbivore, *Spodoptera exigua*, performed worse when feeding from plants previously attacked by both potato aphids and *S. exigua* or aphids only, compared to plants with previous *S. exigua* attack alone. This coincided with a suppression of genes that were originally upregulated by the reciprocal herbivore and with different regulation of plant biochemistry during dual compared to single insect infestation (Rodriguez-Saona *et al.* 2010). Furthermore, Mathur *et al.* (2013) showed that the specialist caterpillar *Plutella xylostella* gained more weight when feeding on plants previously attacked by both *P. xylostella* and *Spodoptera litura* caterpillars than when feeding on plants previously attacked by only *P. xylostella*. However, these studies concerned only two species in conspecific or heterospecific interactions, whereas here we present the effects of two herbivores on responses to a newly arriving third insect species. In addition, the outcome of interactions between species can be herbivore species-specific (Agrawal 2000; van Zandt & Agrawal 2004; Uesugi *et al*. 2013) and density dependent (Kroes *et al.* 2015). For example, initial herbivory of *P. rapae* caterpillars more strongly affected the performance of the subsequently attacking

generalist herbivore *M. brassicae* than that of to the subsequent attacking specialist *P. xylostella* (Poelman *et al*. 2008). It remains to be identified whether the level of specificity in pair-wise herbivore interactions is further modulated in response to multi-herbivore attack or whether specific herbivore species have a prominent effect on the plant phenotype regardless of the presence of other herbivore species (Poelman & Kessler 2016).

Our data provide further insight in how plants physiologically respond to single and dual herbivore attack by analysing the expression of defence genes and levels of the plant hormones SA and JA. Here, we demonstrate that plant resistance differs when induced by multiple insect attack compared to single attack which subsequently affects the performance of successively arriving herbivores. In Kimmeridge plants, 3 d after herbivory by either aphids or caterpillars, SA levels were induced to significantly higher levels compared to plants simultaneously induced by both caterpillars and aphids. Through antagonistic or synergistic crosstalk between JA and SA, plants are able to fine tune their defences (Pieterse *et al.* 2009; Thaler *et al.* 2012a). Although a negative correlation was found between JA and SA levels in milkweed plants after herbivory of both monarch caterpillars and oleander aphids (Agrawal *et al.* 2014), we did not find evidence for overall suppression of JA by SA or *vice versa* (data not shown). However, simultaneous feeding by aphids and caterpillars resulted in a significant increase of JA-dependent *LOX* expression compared with plants infested by only *P. xylostella* caterpillars or control plants. Therefore, differential induction of JA-regulated transcriptional responses to dual insect attack could have mediated a decrease in *M. brassicae* performance because resistance to caterpillars (including *M. brassicae* – van Dam & Oomen 2008) is generally induced by the JA signalling pathway (de Vos *et al.* 2005; Stam *et al.* 2014). The induction of plant defence signalling affected both *P. xylostella* and *B. brassicae* performance. Therefore, JA-mediated responses do not only affect caterpillars but also decrease aphid population growth. We showed that *B. brassicae* aphids induced both JA- and SA-mediated resistance (Moran *et al.* 2002) which may affect aphid performance depending on whether it is feeding alone or simultaneously with caterpillars. Only in Old Harry plants simultaneously infested by aphids and caterpillars, there was a strong correspondence between *PR-1* expression and SA level (positive correlation, $r_s = 0.74$; $P = 0.018$). This indicates that expression of *PR-1* can account for changes in SA level and, therefore, *PR-1* expression can be used as a predictor for SA-mediated induced defences. However, direct correlation of gene expression or hormone levels with herbivore performance is difficult because of the different time scales at which these processes occur (Stam *et al.* 2014).

Still, relatively little is known about long-term effects of herbivory on the kinetics of defence-related gene expression or hormone levels upon multiple herbivory (de Vos *et al.* 2005; Kliebenstein 2014). Underwood (2012) showed that plant resistance responses might last for at least 15 days after herbivory, and had not yet decayed by the time a second herbivore arrived on the plant. However, peaks in defence-related

gene expression might decay much earlier (Vos *et al.* 2013b). We observed that 14 d after herbivory a significant up-regulation of *PR-1* expression occurred after feeding by *P. xylostella* caterpillars only in Kimmeridge plants or after simultaneous feeding of both caterpillars and aphids in Old Harry plants. Similar to our results, it has been found before that *P. xylostella* feeding activates SA signalling in *Arabidopsis* and Chinese cabbage plants (Ehlting *et al.* 2008; Koo *et al.* 2013; Kroes *et al.* 2015). Interestingly, after 14 d the majority of the caterpillars had pupated and, thus, caterpillar feeding had stopped. Elevated expression of the SA-regulated marker gene *PR-1* in Kimmeridge plants 14 d after feeding by caterpillars could indicate priming for enhanced defence or a lag in defence response time to caterpillar attack (see Vos *et al.* 2013b). Another possibility could be that an antagonistic effect of JA on SA-mediated *PR-1* expression diminished from the moment the caterpillars stopped feeding upon pupation. Furthermore, the time herbivores spent feeding may differentially affect defence responses induced by later arriving insects. *Spodoptera litura* was negatively affected by previous dual *P. xylostella* and *S. litura* feeding that started 14 d earlier, but not at earlier or later time points (Mathur *et al.* 2013). Similar to the finding of Mathur *et al.* (2013) that the subsequent herbivore was negatively affected by previous insect feeding depending on the duration of herbivory, we found that *M. brassicae* caterpillars performed worst on plants induced by both *P. xylostella* and *B. brassicae* after 15 d of feeding. This indicates that the length of time first inducers spent on feeding before a subsequent herbivore arrives has an effect on the latter. However, declining plant quality over time cannot be completely excluded.

Plant species vary in their responses to herbivores, even though plant hormones and their cross-regulation are generally regarded as conserved among most of the Angiosperms (Thaler *et al.* 2012a). We observed an interaction effect between plant population and insect treatment, indicating that regulation of responses to insect feeding varies significantly within the same plant species. Differences in responses to herbivory between plant populations (Li *et al.* 2014) or closely related plant species (Johnson & Agrawal 2005; Ali & Agrawal 2014) have been observed before. That *M. brassicae* caterpillars are differentially affected by plant populations confirms previous work (Gols *et al.* 2008b). Performance of *M. brassicae* caterpillars was most negatively affected by Winspit populations, which contain the highest total level of glucosinolates compared to Kimmeridge and Old Harry plants (Gols *et al*. 2008). This suggests that performance of *M. brassicae* caterpillars could also have been negatively affected by differences in nutritional quality between the plant populations. Moreover, seasonal changes within controlled climatic conditions in a greenhouse may cause variation in *Brassica* phenotype (Gols *et al.* 2007), which resembles the variation that we observed among experimental rounds.

In conclusion, by combining ecological and molecular approaches to plant-insect interactions we show links between transcriptomic changes and insect responses. We found that changes in gene expression and phytohormone levels caused by

dual herbivory affected a subsequently arriving third herbivore, as an example of trait-mediated interaction networks that are common in insect communities. Plant-mediated effects of responses to single herbivores are well known to affect community composition season-wide (Kessler & Baldwin 2004; van Zandt & Agrawal 2004; Viswanathan *et al*. 2007; Poelman *et al*. 2010). Our study predicts that each subsequently arriving herbivore on a plant may modulate the plant phenotype and thereby affect the assembly of insects colonizing the individual plant. Moreover, the order of arrival of the first colonizers may thereby have profound effects on the course of the dynamics of the inset community. Therefore, to understand how plant-insect communities are structured, we need to identify how networks of herbivore inducers and responders integrate over time (Utsumi *et al.* 2010; Kliebenstein 2014). Such understanding will help to refine the increasingly complex plant-insect interaction models in which factors such as time course, ecological and molecular changes, multiple interacting insect attackers and plant genotype are important. Identification of these interaction networks will allow for a better understanding of how plants have adapted to multi-herbivore attack in natural ecosystems (Poelman 2015; Poelman & Kessler 2016).

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Chapter 6

Cross-seasonal legacy effects of the arthropod community on plant fitness in perennial plants

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Abstract

Individual species may influence the dynamics of the community they are part of long after they have left, so-called legacy effects. In an insect-plant system, individual herbivores may have legacy effects on the community as a result of the species-specific responses they induce in a plant, which affect subsequent insect colonisation. It is, however, unknown whether these legacy effects persist across years for perennial herbaceous plants. If the legacy of insect communities on plants in the vegetative year affects community assembly on the same plants in reproductive years, this may have consequences for plant fitness.

Here, we studied legacy effects of individual insect herbivores and the arthropod community as a whole within and across years in a two-year field experiment on perennial wild cabbage plants, *Brassica oleracea* L. (Brassicaceae). The arthropod community was monitored on plants that had been induced with either aphids, caterpillars or no herbivores in a full-factorial design across years. We quantified the plant traits height and number of leaves and flowers to understand mechanisms that may mediate legacy effects. Also we evaluated plant fitness consequences of these legacy effects by measuring seed set in the first reproductive season.

We found legacy effects across years on arthropod community composition: variation in carnivore community composition correlated across years. Importantly, herbivore community composition on plants in the vegetative stage affected seed set in the next year. This may have been mediated by plant traits, as height and number of leaves were affected by the herbivore community in the first year. Individual herbivores that were used to induce plants did not leave legacies within or across years, but a specific order of sequential induction by herbivory in two consecutive years did affect plant seed set.

Thus, legacy effects of the insect community in the previous year partially shape the arthropod community composition and affect plant fitness in the next year. We discuss processes that may have driven these legacies, such as the carry-over of insect density, plant genotypic variation and plant phenotypic plasticity. Furthermore, the ecological and evolutionary consequences of legacies in this perennial plant-arthropod system are highlighted.

Keywords

Brassica oleracea, community composition, community dynamics, herbivory, insect-plant interactions, long-term effects, seed set

Introduction

Although the presence of species or interactions among species in ecological communities may be brief, the effect of their presence or their interaction may be traced back over a much longer time course after the initial species or interaction has passed, for example in the trajectory of community assembly. Such legacy effects can have important consequences for later species interactions and community dynamics (Ohgushi 2005; Utsumi *et al.* 2010; Kostenko *et al.* 2012; Wurst & Ohgushi 2015). Long after they have left, individual species may have prominent influences on community organisation when they have a long-term effect on the phenotype of a basal resource that structures communities (Wurst & Ohgushi 2015). For example, root exudates from a plant can influence the soil microbiome and subsequent succession of plant communities (van der Putten 2003; de Deyn *et al.* 2004). The presence of a species can also have a legacy on interactions across several trophic levels, as was shown by changes in a multitrophic community where exclusion of an ungulate affected a tree community and thereby the insect and bird community during a period up to 30 years (Nuttle *et al.* 2011). Furthermore, not only species presence, but also interactions between species can cause legacies, through interaction-induced changes in traits of one or all species involved (Utsumi *et al.* 2010; Wurst & Ohgushi 2015; Ohgushi 2016).

In plant-insect communities, legacies of species interactions are well characterised for species assemblies within a single season (van Zandt & Agrawal 2004b; Poelman *et al.* 2010; Wurst & Ohgushi 2015). To save costs of defence in the absence of herbivores, many plant species only invest in enhanced levels of defence in response to actual herbivore attack (Karban 2011; Mithöfer & Boland 2012; Stam *et al.* 2014). These induced plant responses provide plants with enhanced resistance to the current attacker, but at the same time the induced plant phenotype potentially affects interactions with other community members, resulting in so-called indirect plant-mediated species interactions (Ohgushi 2005; Utsumi *et al.* 2010). Because plant responses to herbivory are often specific for the guild or even herbivore species that attacks the plant (de Vos *et al.* 2005; Schoonhoven *et al.* 2005; Bidart-Bouzat & Kliebenstein 2011; Soler *et al.* 2012a), each herbivore species may differentially affect other community members (Rodriguez-Saona *et al.* 2005; Soler *et al.* 2012a; Mathur *et al.* 2013; Stam *et al.* 2014). Therefore, each herbivore species may have unique legacy effects on the development of the community within the growing season of that plant (van Zandt & Agrawal 2004b; Viswanathan *et al.* 2007; Poelman *et al.* 2010; Wurst & Ohgushi 2015; Stam *et al.* 2016b). These legacy effects by indirect plant-mediated species interactions have been found to also affect reproductive fitness of annual plants (McArt *et al.* 2013) and may thus be important components of plant defence trait evolution (Poelman & Kessler 2016). For the insect community on perennial plants, legacies of insect plant-interactions after the initiating insect has left could play an important role

when the legacy is transferred across growth seasons (Miller-Pierce & Preisser 2012; Wurst & Ohgushi 2015).

In contrast to annuals, perennial plants have multiple growth seasons that typically consist of a distinct vegetative growth season followed by one or multiple years during which the plants flower and reproduce. When legacies of insect-plant interactions in the vegetative season extend across years into reproductive seasons, this may have important consequences for plant fitness (Wurst & Ohgushi 2015). Although induced responses to single herbivores may have season-long legacies on plant-associated insect community composition (van Zandt & Agrawal 2004b; Viswanathan *et al.* 2005; Poelman *et al.* 2010), little is known about how long it takes for induced plant responses to decay, especially in the context of multiple growth seasons of perennial herbs (Underwood 1998; Gomez *et al.* 2010; Karban 2011; Underwood 2012). Several examples show that herbivore-induced plant responses may persist throughout several growth seasons in perennial trees (Haukioja *et al.* 1985; Haukioja 1990; Young & Okello 1998; Nuttle *et al.* 2011; Miller-Pierce & Preisser 2012). This implies that legacy effects of plant-herbivore interactions can extend beyond a single growth season. Thereby, also in temperate regions where winters may cause plant-associated communities to re-assemble each year, long-lasting legacies may connect assemblies across years (Karban 2011; Wurst & Ohgushi 2015). It is thus important to know whether such long-lasting legacies of early herbivores also exist for the insect community associated with perennial herbaceous plants, especially when effects in a vegetative year affect plant reproduction in subsequent years (Wurst & Ohgushi 2015).

Here, we studied two-year legacy effects of early season herbivory on arthropod community composition and the consequences for fitness of an herbaceous perennial plant. In a field experiment over two consecutive years, wild perennial *Brassica oleracea* plants were inoculated early in the season with either of two specialist insect herbivore species from different feeding guilds (aphid or caterpillar), or no herbivore (control), in a full-factorial design across years. Arthropod community composition was monitored in the vegetative and first reproductive season, and at the end of the second year seed set was measured. Specifically, we address the following questions: i) Does early-season herbivory by aphids or caterpillars affect arthropod community composition and plant fitness, either within or across years? ii) Does variation in arthropod community composition as a whole cause legacy effects on community composition and plant fitness, either within or across years? and iii) Which insect species and which plant performance traits are involved in the above processes? We discuss the data in the context of insect-plant ecology and evolution.

Materials and methods

Field site

Herbaceous wild perennial *Brassica oleracea* L. (Brassicaceae) plants, originating from Kimmeridge, Dorset, UK (50°36'N, 2°07'W) (Gols *et al.* 2008b) were planted in a common garden in the vicinity of Wageningen University, the Netherlands. Seeds that had earlier been collected from approximately twenty plants of the Kimmeridge population were sown in mid-April 2012 and transplanted to peat soil cubes 11 d later. Seedlings were grown in a greenhouse until 4 w after sowing, after which they were placed outside to habituate them to field conditions. In week 21 (end of May) 2012, 72 plots of 12 plants each in a 4 x 4 square (omitting the central four plants to ensure equal plant neighbour effects) were established in the field. Within-plot planting distance between plants was 1 m while distance between plots was 4 m. The gap between plots was sown with a *Poa*/*Lolium* grass mixture. To ensure a uniform edge environment, two rows with plants of the annual *Brassica nigra* were planted at 4 m distance from the plots at the border of the field, 1 m between rows and 0.5 m between plants within rows. The seeds for these plants had been collected from wild *B. nigra* plants in the vicinity of Wageningen, the Netherlands, and were sown and treated similar to the *B. oleracea* plants as described above. Plots and edge were regularly manually weeded and grass paths were regularly mown. The plants were experimentally inoculated with herbivores early in the season of two subsequent years (2012 and 2013, see below) and exposed to naturally occurring arthropods during the rest of season in the two years. In the winter period (January 8 – April 3 2013), plants were protected from severe freezing/dehydrating conditions by covering the whole *B. oleracea* field with a cloth (26 g m^{-2}, AMEVO, the Netherlands). *Brassica nigra* plants were re-sown and planted next spring, similar as described above (planting in the field in week 21, end of May 2013).

Legacy effects: two-year common garden experiment

In order to study legacy effects of herbivore induction on community composition, and legacy effects of variation in the community as a whole, we manipulated the first herbivores arriving on the individual *B. oleracea* plants. Six days after planting, the plants were inoculated with either aphids, caterpillars or left uninfested. Cabbage aphids *Brevicoryne brassicae* L. (Hemiptera: Aphididae) and diamondback moth caterpillars *Plutella xylostella* L. (Lepidoptera: Yponomeutidae), both specialists on brassicaceous plants, were obtained from the stock rearing at Wageningen University. These insects were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv Cyrus) under greenhouse conditions (21 ±1 °C, 50-70 % relative humidity, 16L : 8D cycle).

In a full-factorial setup, plants were exposed to all nine possible combinations of

either aphids, caterpillars or no insects in the two consecutive years (Table 1). All plants within one plot received the same treatment, and each treatment had 8 replicates (plots), randomly distributed over the field. In week 22 of 2012 (end of May) and week 20 of 2013 (mid-May), we applied one of the induction treatments to all 12 plants in a plot. On a fully-expanded leaf we carefully inoculated using a small brush either i) five *B. brassicae* aphids in a mixture of adults and 4th instar nymphs (A), ii) three *P. xylostella* caterpillars in 2nd larval stage (C), or iii) no herbivores (N). In 2012, only 3 of the in total 9 treatments over the two-year experiment were monitored, which each had received different early-season herbivory treatments in the first year and no herbivory in the second year (AN, CN, NN). We had to restrict the number of observations in this year to allow for intensive monitoring of the within-year effects of early season herbivory on a detailed time frame (twelve moments of monitoring). We assumed that this subset of plots was representative for the full sweep of replicates of early-season herbivory treatments in the first year. In 2013, the 72 plots with all 9 treatments were monitored, although with a lower frequency (two moments of monitoring), to focus on across-year effects. To test for legacy effects on community composition within and across years, one week after induction in either year the monitoring of the arthropod community started. We followed community development by repeatedly sampling the same four plants per plot that were randomly selected during the first round of observations. In 2012, we collected data over twelve time points during the season on a weekly basis. In 2013, we selected four new plants per plot that showed flower buds in week 20 (non-flowering plants were excluded from monitoring). The community on these plants were monitored two times, during early season (week 21 – 25) and mid/late season (week 25-33). Within each of the two time periods, plots with different treatments were monitored in a random order to minimize time-effects on the community composition. All 72 plots which now had received the full factorial 3x3 design of 9 different treatment combinations of early herbivore inoculation over both years, were monitored for all occurring arthropod species as described below.

Monitoring plants for arthropods in both years occurred through visually screening for all visible life stages of all living insects and other invertebrates such as spiders and slugs (here collectively referred to as 'arthropods') on the upper- and lower parts of each leaf. Both herbivores and carnivores (predators plus parasitoids) were recorded. However, fast-flying insects, such as adult parasitoids, adult lepidopterans and pollinators were not recorded as their presence could not be accurately assigned to a single plant. Parasitized aphids ('mummies') were identified as aphid parasitoids; parasitoids of lepidopterans were identified by their cocoons once emerged from their caterpillar host in the field. The number of individuals per species was recorded per plant, taking all life stages together. See Supplementary Table 1 of Chapter 4 for a list of observed arthropod species.

To test which plant performance parameters corresponded with insect community development, we also recorded plant total height and number of leaves in both

years at the same moments when plants were monitored for arthropods (twelve times in 2012 and two times in 2013). In addition, in the reproductive season (2013) the number of flower racemes (unbranched stalks bearing flowers) was recorded at the two same time points.

During the intermediate winter period October 2012 – March 2013, a few randomly selected plants in the field were screened for the presence of arthropods, but none were found.

Seed harvest

To test whether either early season herbivory treatments or total arthropod community composition exerted legacy effects on plant fitness, seeds of all monitored plants were harvested. Seeds formed during the first plant reproductive season (2013) were collected after the second period of monitoring, from week 34 to the beginning of week 39. Racemes with dry seed pods were cut and placed in a paper bag per plant. A cloth underneath the plant collected seeds falling during the harvesting process; all fallen seeds per plant were included in the bag. Plant material further dried while stored until processing, during which seeds were separated from remaining plant material. The number of seeds per plant was computed by dividing the total weight of the seed batch by the weight of 100 counted seeds, and multiplied by 100.

Statistical analyses

Early-season herbivory effects on community composition

We first tested the effects of early-season herbivory treatments (aphids, caterpillars or none) on the herbivore or carnivore community composition within and across years on plot level, averaging the abundance of each species over the four plants per plot. For the first year (2012), abundance of each species was cumulated over all 12 monitored time points to obtain for each plot the community composition over the whole season. Only the 24 plots that were monitored that year were used for this ('M', Table 1). For the second year (2013), abundance of each species was similarly cumulated over the two time periods, and all 72 plots were used (Table 1), except for plots in which all plants had died (n=4). Redundancy analyses (RDAs) were used to test the effects of the early-season herbivory treatment applied to a plot (aphid, caterpillar or none) in the first or second year, on the community composition per plot (cumulated species abundance) in the first or second year. Tests were performed with a Monte Carlo permutation test with 499 unrestricted permutations. A linear method was assumed valid as the length of species data gradient was less than 3 turnover (SD) units long (Šmilauer & Lepš 2014). Species numbers were log (y+0.25) transformed prior to analyses. In these analyses, abundances of *P. xylostella* and *B.*

brassicae were made supplementary (excluding them from ordination analysis, but projecting them afterwards in biplots) to exclude effects of herbivores that were directly manipulated by our treatments.

Early-season herbivory effects on plant fitness

First, to test the effects of early-season herbivory on plant fitness in either the first or the second year and the interaction between both years, the number of seeds per individual plant were analysed by two-way ANOVA. Second, to assess in more detail which combinations of species in first-year herbivore inoculation – second-year herbivore inoculation specifically affected plant fitness, we conducted a one-way ANOVA on all nine treatment combinations (Table 1). Third, we grouped treatments that had the same herbivore inoculation (aphid, caterpillar or none) in the first year, or the same herbivore inoculation in the second year, but that differed in herbivore species in the following or previous year respectively (Table 1). On each of these six groups we conducted a one-way ANOVA, followed by an LSD post-hoc test if effects were significant. For all tests, seed set of individual plants from all monitored plots in 2012 and 2013 were used (Table 1), except for plants that had died before they produced seeds (n=52 of 288 monitored plants). Number of seeds per plant was double square-root transformed to meet assumptions of normal distribution and homogeneity. ANOVA tests were carried out in SPSS Statistics for Windows, Version 22.0.0.1 (Armonk, NY, USA; IBM corp.).

Table 1. Early-season herbivory treatments in two consecutive years, applied in a common-garden field experiment on wild perennial *B. oleracea* plants. '**M**' indicates treatment plots that were monitored in both 2012 and 2013; the other plots were monitored in 2013 only.

Early-season herbivore year 1 (2012)	**Early-season herbivore year 2 (2013)**	**Abbreviation**
Aphids *B. brassicae*	Aphids *B. brassicae*	AA
Aphids *B. brassicae*	Caterpillars *P. xylostella*	AC
Aphids *B. brassicae*, **M**	No early-season herbivore	AN
Caterpillars *P. xylostella*	Aphids *B. brassicae*	CA
Caterpillars *P. xylostella*	Caterpillars *P. xylostella*	CC
Caterpillars *P. xylostella*, **M**	No early-season herbivore	CN
No early-season herbivore	Aphids *B. brassicae*	NA
No early-season herbivore	Caterpillars *P. xylostella*	NC
No early-season herbivore, **M**	No early-season herbivore	NN

Community legacy effects within and across years: structural equation model

To relate early-season herbivory treatments, herbivore and carnivore community composition in either year and seed set of the perennial plants, we used Structural Equation Modelling (SEM). The produced SEM was used to address two questions: i) whether early-season herbivory inoculations affected community ordination and plant fitness within and across two seasons, and ii) whether variation in the composition of the herbivore and carnivore community affected community composition and plant fitness within and across years.

To obtain one value per plot for each of the variables, arthropod community composition was represented by ordination scores of field plots on the first axis of a Principal Component Analysis (PCA; see Supplementary material for additional note). For PCAs, herbivore and carnivore community data in 2012 and 2013 were similarly prepared as described above for RDAs, except that for all variables, only the 24 plots were used that were monitored for community composition in both 2012 and 2013 ('M' in Table 1). Two plots of which all plants had died in 2013 were excluded. Species data were log (y+0.25) transformed prior to PCA, with abundance of *P. xylostella* and *B. brassicae* made supplementary. The resulting ordination score on the first principal component axis of each field plot was used as input for the SEM. Herbivory treatments in 2012 were included as either aphid, caterpillar, or none. Because the selected plots all received the treatment 'none' in 2013 (Table 1), early season herbivory treatment of 2013 was not included in SEM. Average seed set per plot for SEM were square-root transformed prior to analysis to meet assumptions of normality of SEM. The model best fitting the data was selected by removing non-significant paths from the model. In SEM, the goodness of fit of the model is assessed by comparing the observed and model-predicted covariances with a χ^2 test. The model is acceptable (there is reasonable fit between model and the data) when the χ^2 values have an associated *P*-value of > 0.05 (Grace 2006). SEM analyses were carried out with 'sem' package in R (version 3.0.1, R Development Core Team 2013).

Ordination of species involved in legacy effects: principal component analyses

Species ordination plots were made to obtain more detailed information on which individual species were involved in legacy effects to community composition and plant fitness. For the two most interesting paths of the SEM described above (see Results), scatterplots were made indicating which species likely occur in the same plot (e.g. long species-arrows pointing in the same direction). First, to show the relationship of carnivore species occurrence in plots in the first and second year, two scatterplots of carnivore species in either year were made by PCA as described above, using the same data as was used for SEM input (for these original scatterplots, see Supplementary material). The two scatterplots were then overlaid, such that the carnivore species ordination of both years was depicted in one image

(See Supplementary material for justification of this method).

Second, to show the relationship of the herbivore species present in the first year with seed set of those plants in the following year, another PCA biplot was made depicting ordination of herbivore species in the vegetative season (2012) and seed set in the reproductive season (2013). Seed set in 2013 was a supplementary variable that does not influence species ordination. Afterwards an arrow indicating the direction of plots with increasing seed set was projected onto the herbivore species scatterplot. (The RDA biplots shown in the supplementary material for herbivore community 2012 + seed set; carnivore community 2012 + seed set; and carnivore community 2013 + seed set were similarly obtained.)

All ordination analyses (RDA, PCA) were executed with Canoco 5.04 for Windows (ter Braak & Šmilauer 2012). See Supplementary material for more details on interpretation of ordination plots using the biplot rule.

Plant performance traits involved in herbivore-community legacy effect on plant fitness: structural equation model

We performed another SEM to investigate whether and how plant performance traits mediated first-year herbivore community legacy to seed set of the second, reproductive year. Herbivore community in 2012 was represented by PCA scores similar to those in the first SEM. We used the plant traits 'height' and 'number of leaves' in both years as indicators of plant size, and number of flower racemes in 2013 as indicator of amount of reproductive tissue. For plant traits in 2012, measurements during the peak of the arthropod season (week 35; Stam *et al.* 2016b) were taken as input. For plant traits in 2013 the situation was different due to a reduction of number of leaves while the plants started flowering and subsequently formed seeds as the season progressed. Therefore, in 2013 measurements when plant traits were on average at their maximum value were taken as input: early season (week 21-25) for number of leaves; mid/late season (week 25-33) for plant height and number of flower racemes. Number of leaves in 2013 was square-root transformed to obtain normality. Similar as for the first SEM on community legacy effects, average seed set per plot in 2013 was used and data were square-root transformed for normality.

Results

Early-season herbivory effects on community composition and plant fitness

Early-season herbivory treatments in the first and second season had no effect on herbivore or carnivore community composition either within the year or in the following year, although early season herbivory in the first year had a near-significant effect on the carnivore community composition in the following year

Table 2. Results of Redundancy analysis (RDA) of early season herbivory treatments in either year on wild perennial *B. oleracea* plants, to the herbivore and carnivore community composition within and across years. Treatments applied early in the season in 2012 and 2013 were either aphids *B. brassicae*, caterpillars *P. xylostella* or no herbivory. Percentages show the % cumulative explained variation by the first two RDA axes; F-values are pseudo-F values of Monte Carlo Permutation test with 499 unrestricted permutations. Degrees of freedom of explanatory variables was in all cases 2; $\alpha = 0.05$.

Year early herbivory	Affected Community	%	F	P
2012	Herbivores 2012	6.82	0.8	0.758
2012	Carnivores 2012	8.94	1.0	0.422
2012	Herbivores 2013	2.00	0.7	0.814
2012	Carnivores 2013	6.02	2.1	0.056
2013	Herbivores 2013	3.29	1.1	0.374
2013	Carnivores 2013	1.40	0.5	0.828

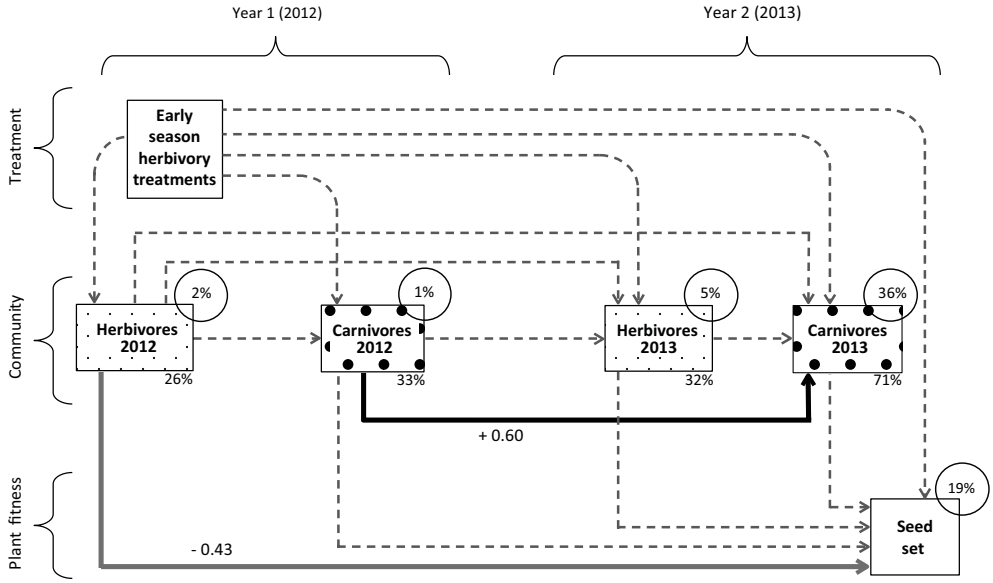

Figure 1. Structural Equation Model (SEM) of relations between early-season herbivory treatments in the first year (2012), herbivore and carnivore community composition in the first (2012) and second year (2013) and seed set of plants in the second year (2013). For community composition, scores on the first ordination axis of Principal Component Analysis (PCA) were used, of which the % explained variation is mentioned below each block. R^2 values (%) as shown on top of each endogenous explanatory variable (circle) give the explained variation by all paths to that variable by the SEM. Dotted lines indicate non-significant effects, while continuous lines show significant effects, with their standardized path coefficients: black line for a positive relationship, grey line for a negative relationship.

(P=0.056, Table 2). Also, in the SEM on community legacy effects (see below, Figure 1), none of the paths significantly related early-season herbivory treatments in the first year to herbivore or carnivore community in either year, nor to plant seed set.

Although seed set of individual plants was not affected by early-season aphid, caterpillar or no herbivore feeding in neither the first, vegetative year (ANOVA: F_2:0.317, P=0.729), nor in the following, reproductive year (ANOVA: F_2:1.068, P=0.345), the interaction between herbivore treatments applied in the two years did significantly affect seed set (ANOVA: F_4:2.600, P=0.037). All nine combinations of subsequent early-season aphids, caterpillars or none in the first and in the second year did not seem to differ in their effect on seed set (ANOVA: F_8:1.684, P=0.103), but when early season herbivory treatments were grouped to the same early herbivore species in either the plant vegetative phase or reproductive phase, a more clear pattern appeared (Figure 2). Only when in the first year caterpillars were placed on the plants, seed set of individual plants differed between treatments applied in the second year: plants sequentially induced with caterpillars in both years (CC) had fewer seeds than plants that had received caterpillars in the first year and aphids in the next year (CA); while seed set of plants infested with caterpillars followed by no early-season herbivory was intermediate (CN; Figure 2). Plant seed production was thus determined by an interaction of early herbivore presence in the vegetative year and following first year of flowering, rather than determined by the early herbivore attack in the flowering year alone.

Legacy of whole arthropod community within or across years

Although our early-season herbivory treatments did not affect community composition in the same or next year (above), herbivore and carnivore community composition in both the vegetative year (2012) and first flowering year (2013) did reveal legacy effects of total communities across years. A SEM that included herbivore and carnivore community composition provided a good fit to the data, and showed that especially the carnivore community was shaped by legacy effects (whole-model fit: X^2_{10}=6.531, P=0.769). Carnivore composition in the first year had a significant effect on the carnivore composition in the following year (SEM, z:3.43, P<0.001, Figure 1). This was not mediated by herbivore community within or across years, as none of those paths were significant (Figure 1). Also, the near-significant effect of early-season herbivory to carnivore community composition across years (Table 2) was not seen back in this SEM (Figure 1).

Second, herbivore community composition in the plant vegetative season (2012) had a direct effect on seed set in the following, reproductive season of the plants (2013) (SEM, z:-2.21, P=0.027). Also this was not mediated by either herbivore or carnivore community composition within or across years, as none of the

intermediate paths were significant (Figure 1). Thus, mainly across-year effects, rather than within-year effects, influenced carnivore community composition and plant fitness.

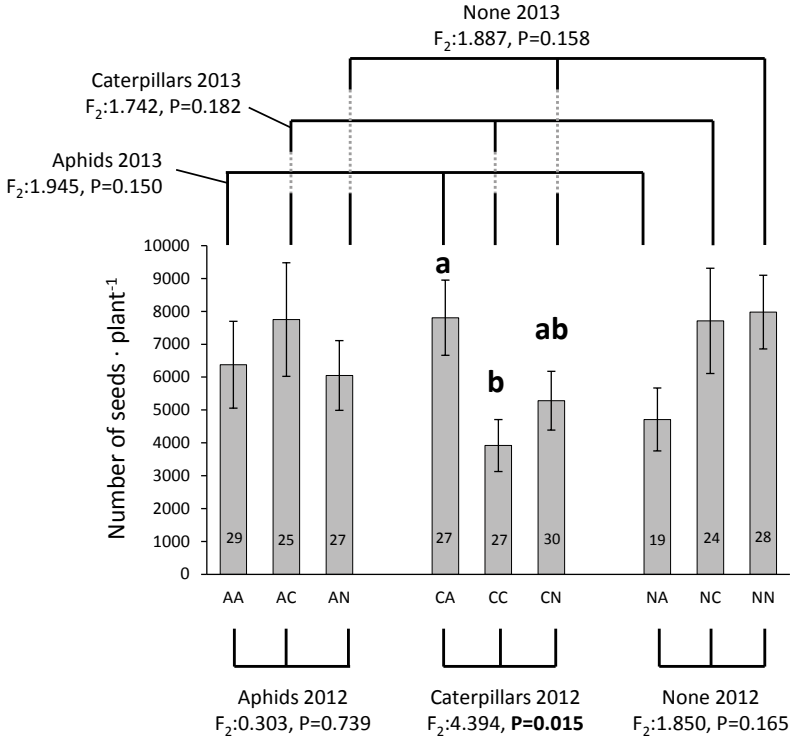

Figure 2. Average seed set (±SE) per plant in 2013 of wild perennial *B. oleracea* plants was affected by sequential early season herbivore treatments in two consecutive years. Abbreviations for early-season herbivore inoculation: A: aphids *Brevicoryne brassicae*, C: caterpillars *Plutella xylostella*, N: no herbivores (None); the first letter indicates inoculation in the first year (2012) and the second letter inoculation in the second year (2013). As the interaction between herbivory treatments in 2012 * herbivory treatments in 2013 was significant, additional ANOVA tests were performed to gain insight which sequence of early herbivory in 2012-2013 affected seed set. Treatments were grouped to have the same herbivore inoculation in the first year (2012), or the same herbivore inoculation in the second year (2013), but that differed in herbivore species in the following or previous year respectively. ANOVA results for each group are shown; bold P-value indicates a significant difference in number of seeds between treatments tested ($\alpha = 0.05$). Different letters above the bars indicate significant different numbers of seeds between treatments in that group. Number of replicates per treatment are shown within each bar.

Arthropod species involved in community legacy effects

Especially parasitoids were involved in mediating legacies of the first year-carnivore community to the second year-carnivore community (Figure 3a, Supplementary Table 1 of Chapter 4). For example, parasitoids associated with the caterpillar *P. xylostella* likely occurred in the same plots in high abundances in both years (e.g. long arrows of the same species in either year pointing in the same direction in the PCA plot). The other way around, parasitoids of, for example, the aphid *B. brassicae* or the parasitoid *Cotesia rubecula* likely occurred in the same plots with a high abundance in one year, but with a low abundance in the second year (or *vice versa*, e.g. long arrows pointing in opposite directions in the PCA plot). The abundances of predators belonging to e.g. the Neuroptera or Syrphidae larvae, however, did not show a strong correlation across years (e.g. arrows almost perpendicular to each other; Figure 3a), although spiders (*Araneae*) and ladybeetles (*Coccinellidae*) showed positive or negative relations across years respectively, but only with arrows of small to intermediate length.

The abundance of some specific herbivore species that were present in plots in the vegetative year corresponded with plant fitness a year later (Figure 3b). Numbers of the generalist caterpillar *Mamestra brassicae*, *Aleyrodes* spp. whiteflies, and the specialist aphid *B. brassicae* on vegetative plants coincided with high seed set in the following year. On the other hand, species that were abundant in plots with a low seed set the next year, such as the two flea beetle species (*Phyllotreta undulata* and *P. atra*) and aphids other than *B. brassicae* or *Myzus persicae*, explained plant fitness less well (e.g. species arrows pointing in opposite direction from seed set are smaller than those pointing in the same direction as seed set, Figure 3b). Thus, mainly species whose abundance in a plot was positively correlated with the number of seeds caused a legacy across years.

Community legacy to plant fitness mediated by plant performance traits

The plant performance traits we had measured could have been involved in mediating the above observed legacy effects of the herbivore community in the vegetative season to plant fitness in the following reproductive season (Figure 4). The SEM had good data fit (X^2_{15}=21.988, P=0.108) and revealed that herbivore community composition in the first year significantly affected plant height (SEM, z:-2.14, P=0.032) and number of leaves (SEM, z:-3.23, P=0.001) within the first year (2012). A near-significant path was found for the first year-herbivore community affecting the number of flower racemes in the next year (SEM, z:-1.93, P=0.054). Subsequently, the number of flower racemes in the second season was positively correlated with the number of seeds in that year (SEM, z:3.28, P=0.001). However, plant performance traits (height, number of leaves) did not correlate with each other across the two years. In conclusion, herbivore community in 2012 could have affected seed set in 2013 indirectly through plant performance traits, although the

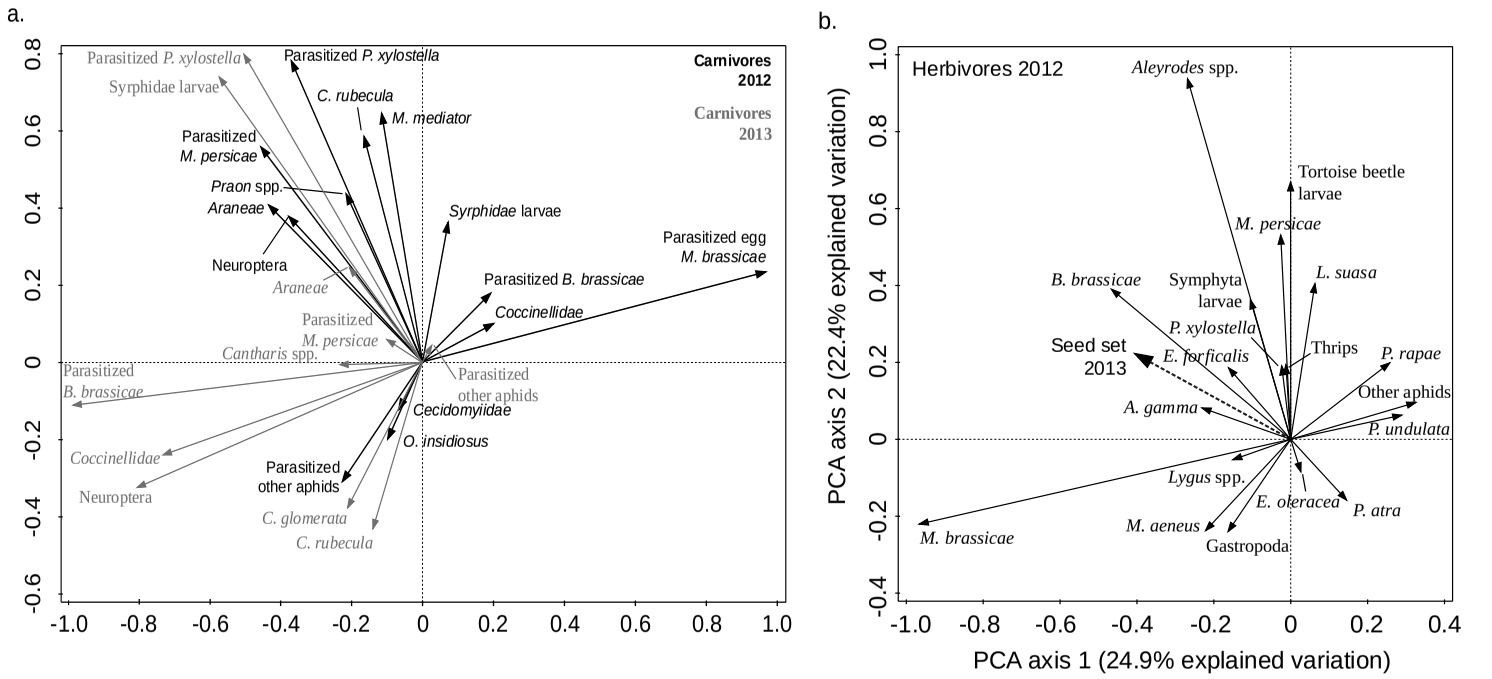

Figure 3. Principal Component Analysis (PCA) biplots showing species ordinations of a) carnivore community in 2012 (black arrows) and 2013 (grey arrows; one combined overlay plot) and b) biplot of herbivore community in 2012 with variable 'Seeds' (seed set 2013). Input variables for PCA were same as used for SEM. Long arrows pointing in the same direction show a positive correlation, whereas long arrows pointing in opposite directions show a negative correlation. Short arrows indicate a weak effect (low amount of explained variation). As a) is an overlay of two individual PCA scatterplots (see Supplementary material), the % explained variation of each axis could not be given. In PCA biplot b), seed set explained 6.6% of the variation in the herbivore community composition. For a list of species, see Supplementary Table 1 of Chapter 4.

connecting chain of mediating plant traits was just not significant. In this SEM where plant traits are included, the direct path from the herbivore community to seed set in the next year represents all other traits beyond our measurements. This path is here not significant anymore, compared to the SEM where plant traits were not included (Figure 1). This indicates that, next to other possible traits, the traits we measured are important, although they could not explain all variation in seed set.

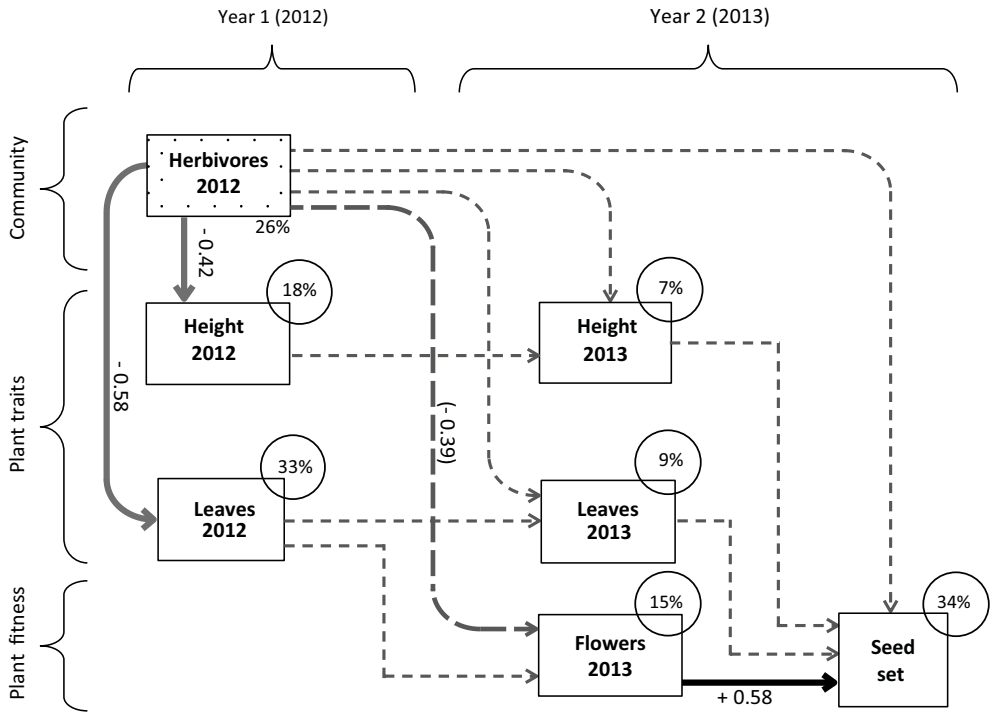

Figure 4. Structural Equation Model (SEM) of relations between herbivore community composition in the first year (2012), plant traits in the first, vegetative year (2012) (maximum height and number of leaves) and second, reproductive year (2013) (maximum height, number of leaves and number of flower racemes), and seed set of plants in the second, reproductive year (2013). For herbivore community composition, scores on the first ordination axis of Principal Component Analysis (PCA) were used, of which the % explained variation is mentioned below the block of herbivores 2012. R^2 values (%) as shown on top of each endogenous explanatory variable (circle) give the explained variation by all paths to that variable by the SEM. Dotted lines indicate non-significant effects, while continuous lines show significant effects, with their standardized path coefficients: black line for a positive relationship, grey line for a negative relationship. The path from herbivore community in 2012 to the number of flower racemes in 2013 was a near-significant (P=0.054) negative relationship.

Discussion

In a common garden experiment executed over two years, we found community legacy effects in a perennial plant-arthropod system: variation in carnivore community composition exhibited legacy effects on the carnivore community composition in the following year. Importantly, herbivore community composition in the plants' vegetative growth season had legacy effects on plant fitness in the next reproductive year, and our data hint that this was mediated by plant performance traits in the vegetative phase. Herbivore community composition in the vegetative growth season affected plant size and eventually seed set in the next season. In addition to the plant performance traits measured here, it is likely that other plant traits such as those involved in herbivore defence or attraction of pollinators are involved as well. In contrast to our expectations, experimentally inoculated early-season herbivores did not leave legacy effects in community composition of either year, but a specific order of sequential early-season herbivory in two consecutive years affected plant seed set. Thus, composition of the arthropod community associated with the perennial plant *B. oleracea* is to some extent shaped by legacy effects on arthropod community organisation across years and mediated by plant-performance traits with long-lasting consequences for plant fitness. The presence of these legacy effects is likely integrated in plant defence and reproduction strategies of perennial plants.

Processes mediating plant-arthropod legacy effects within and across years

There are several processes that could mediate insect-plant legacy effects across years, affecting both the arthropod community (e.g. community composition) and the plant (e.g. plant phenotype and fitness) (Wurst & Ohgushi 2015). Here, we discuss three of these processes, namely carry-over of insect densities across years, the role of plant genotypic variation and plant phenotypic plasticity within and across years.

Arthropod density-dependent effects across years

Legacies of early to later season community composition may have occurred through insect density-dependent effects, either via offspring of individual community members, or via trophic cascades (Townsend *et al.* 2003; Schmitz *et al.* 2004). Here, we did not find evidence for trophic cascades as none of the paths between herbivores and carnivores in our SEM models were significant. However, in our analyses at the community level, we may have overlooked herbivore-carnivore interactions at the individual species level. Apart from trophic interactions, also pollinators may have played a role in mediating the effects of the herbivore community to seed set in the following year. In their larval stage, pollinators can be part of the herbivore (e.g. Lepidoptera) or carnivore (e.g. Syrphidae) community, thereby creating legacy

effects through ontogenetic niche shifts (Wurst & Ohgushi 2015). Finally, arthropod species may have overwintered on, under or near their host plant. Emergence in the following spring on the same plant could then cause correlations in community across two years. However, this seems unlikely as many insects are highly mobile upon emergence in spring (Schoonhoven *et al.* 2005). Also, we did not observe any overwintering insects, although small hidden life stages such as eggs or pupae may have been overlooked, especially those that hibernate in the soil.

Plant genotype effects across years

Plant genotype may also have played a role in the observed legacy effects (Bukovinszky *et al.* 2008; Bálint *et al.* 2015). Plants with the same genetic background harbour a similar associated arthropod community (Bangert *et al.* 2006; Whitham *et al.* 2006; Keith *et al.* 2010; Meneses *et al.* 2012). This could explain why the carnivore communities per plot were related across the two years. Wild *B. oleracea* plants show even within plant populations a large variation in various traits (Lannér-Herrera *et al.* 1996; Raybould *et al.* 1999), such as plant chemistry (Gols *et al.* 2008b), including the volatiles they emit upon herbivory (Gols *et al.* 2011). Carnivore species, especially parasitoids, may have selected similar host-plant genotypes in each of the two years because they were emitting similar cues in either year (Harvey & Gols 2011b; Gols *et al.* 2012). Why herbivores, that have a direct trophic link with plants, did not seem to respond to the same plant genotypes across years, is inconclusive from our results.

Plant phenotypic plasticity within and across years

Plant phenotypic responses to herbivory could have mediated legacies in different ways within and across seasons. Within the season, a chain of short-term induced plant phenotypic changes could have mediated interactions between herbivores that are temporally separated (Ohgushi 2005; Utsumi *et al.* 2010). Such plant traits would need to be herbivore-inducible, affect the colonisation probability of other arthropods on the plant, and last long enough for other arthropods to be affected by and modify these traits in their turn. Various traits would qualify, such as changes in primary and secondary metabolism, including emitted volatiles, or changes in architecture such as branching or density of trichomes (Underwood 1998; Underwood 2012; Wurst & Ohgushi 2015). Moreover, in several plant-insect systems it has been shown that herbivore-induced plant responses affected the arthropod community later in the season (Rieske & Raffa 1998; van Zandt & Agrawal 2004b; Viswanathan *et al.* 2005; Poelman *et al.* 2010; Stam *et al.* 2016b). In our study, plant performance traits such as size in the vegetative season were affected by the herbivore community. These traits are likely to have subsequently affected the occurrence of other species on the plant (Ohgushi 2005; Carmona *et*

al. 2011; Ohgushi 2016). When these effects last until after the herbivores that induced the change have left, such plant phenotypic responses could indeed play a role in mediating legacy effects within the season (Utsumi *et al.* 2010; Wurst & Ohgushi 2015).

Across seasons, however, there may be a gap in herbivore presence during the winter period, such as in our study system. Arthropod-induced plant traits mediating legacies across seasons thus should remain long enough to still affect next season's arthropod community (Haukioja 1990; Wurst & Ohgushi 2015). However, maintaining a response for a long time period of months or years may be costly (Gomez *et al.* 2010; Karban 2011; Vos *et al.* 2013a). The decay time of induced plant responses can last for up to 20 (Underwood 2012) or 28 days (Gomez *et al.* 2010), but further studies of long-lasting plant trait changes are needed (Karban 2011; Underwood 2012), especially for those traits that could last across years (e.g. Haukioja *et al.* 1985). The plant performance traits we measured (height and number of leaves) were not correlated across years in our model, perhaps because the measurements in either year represented different traits. Height in the vegetative phase is a proximate for the amount of photosynthetic tissue, while height in the reproductive year is a proximate for the amount of reproductive tissue because the length of the flower racemes predominantly determined total plant height (Pérez-Harguindeguy *et al.* 2013). However, these plant traits did play an important role in transferring the legacy of herbivores to plant fitness across seasons, because when they were included in the second SEM, some of the indirect paths via plant traits were significant or nearly significant; while the direct path from the herbivore community in 2012 to seed set, which encompasses other traits that we did not measure, was not significant anymore. A possible scenario is that herbivores in the first season could have affected biomass of the vegetative plant, which in its turn may have influenced the number of flowers and seeds in the following year. Yet, other long-term induced plant traits beyond our measurements are likely to have been involved as well, as our measurements did not explain all variation in seed set. For example immobile defence compounds, mechanical defences such as thorns or trichomes, or nutrient allocation could have been involved (Wurst & Ohgushi 2015). Also, priming of plant phenotypic changes could be a possible mechanism (Wurst & Ohgushi 2015). The primed plant state due to previous attack could bridge a gap of inducer absence and transfer the legacy of a previous induction event over a long period without the plant having to pay for maintaining high levels of defence (Galis *et al.* 2009; Vos *et al.* 2013a; Girón-Calva *et al.* 2014; Wurst & Ohgushi 2015).

Integration of legacy-mediating processes

Various processes in insect-plant ecology that may mediate legacy effects can be intrinsically linked or work at different time scales. For example, when a first attacker on a plant seedling induces plant responses that have season-long effects, these

herbivores may determine the course of insect community assembly in that year and also these induced changes in community composition in turn may carry over across years (Viswanathan *et al.* 2007; Stam *et al.* 2014). These effects may be modified by plant ontogenetic changes in (induced) defence that have major consequences for interactions with insects, especially in perennial plants with separated vegetative and reproductive seasons (Boege & Marquis 2005; Underwood 2012). Despite this, our study revealed arthropod community legacy effects from one year to the next.

The different processes mediating legacies may also enhance each other. As an extension of the genetic similarity rule (Bangert *et al.* 2006; Meneses *et al.* 2012) for instance, a herbivore-induced change in phenotype additive to genetic background of an individual plant can further diverge the phenotypes between individual plants and in this way enhance long-term legacy effects (Whitham *et al.* 2003; Johnson *et al.* 2006). In line with this, our study identified that a specific sequence of early-season herbivore inoculation in two subsequent years affected plant fitness. Such repeated application of inducing herbivores may have diverged plant phenotypes and eventually fitness through a combination of effects of variation in plant genotype and herbivore-induced plant phenotypes (Erb *et al.* 2011; Ali & Agrawal 2014; Stam *et al.* 2016b).

Ecological and evolutionary consequences of legacy effects

Legacies in which the effect of an inducing organism persists after the inducing organism is gone (Wurst & Ohgushi 2015) are widely known in ecology. For example, the effects of the presence of an organism, such as a tree with varying tannin levels, extended until after its death where it still affected a community of decomposers through its leaf litter (Whitham *et al.* 2012). More complex, the interaction between a caterpillar and a plant was transmitted *via* the soil community to performance of another caterpillar and its parasitoid when they were feeding on a plant that later grew in the same soil (Kostenko *et al.* 2012). In addition to such effects on a local scale, ecosystem engineers varying from insects (Barton *et al.* 2014) to elephants (Pringle 2008) can affect whole landscapes and their associated organisms through the changes they make in their environment (Schoonhoven *et al.* 2005; Nuttle *et al.* 2011). Here, we show that not only individuals, but also the composition of a plant-arthropod community including the interactions between community members can have legacy effects. These community legacies in our study persisted across years on a perennial herbaceous plant, a phenomenon that has previously been described predominantly in woody plants (Haukioja *et al.* 1985; Young & Okello 1998; Nuttle *et al.* 2011; Miller-Pierce & Preisser 2012). Moreover, we mostly found legacies across years and not within years. These legacy effects may play a prominent role in ecosystem organisation and functioning as plant-arthropod community composition influences large-scale processes such as associations between plants and insects, which subsequently influence for example birds (Nuttle *et al.* 2011);

ecosystem services; and nutrient cycling (van der Putten 2003; Whitham *et al.* 2012; Wurst & Ohgushi 2015).

Moreover, short- and long-term legacy effects may shape the landscape of natural selection. Plant-mediated legacy effects of herbivory on the insect community can affect selection pressure on plant traits involved in defence or tolerance as these traits may influence plant fitness (Boege & Marquis 2005; Utsumi 2011; Ohgushi 2016). Such community-induced or -selected traits subsequently feed back to insect community organisation, but also to trait composition of these insects (Ohgushi 2016). It remains to be identified which insect herbivore species leave legacies that feed back to selection on plant traits (Poelman & Kessler 2016). An herbivore or herbivore community that exerts a direct effect on plant fitness can pose selection pressure on plant reproductive strategies, for example on perennial plants that vary their reproductive investment across years to adapt to insect herbivore pressure (Boege & Marquis 2005; Xiao *et al.* 2013). In conclusion, an arthropod community can have long-term consequences that persist until after the interactions between individuals has ceased, for both the arthropod community composition itself as well as for plant fitness and possibly plant trait selection.

Future perspectives

Long-lasting legacies of individuals or whole communities may have far-reaching consequences for plant-insect interaction outcomes and fitness of the species involved. Yet, to understand the mechanisms underlying these legacies, we need to further elucidate the underlying traits that mediate them and which function they play in structuring insect communities.

Moreover, the plasticity of plant phenotype during a chain of short-term induced changes or the maintenance of a response during a long-term decay trajectory may be limited (Underwood 1998; Karban 2011; Underwood 2012). Therefore, we need to know the plant physiological limitations and costs of potential plant traits that could mediate long-term arthropod legacies (Boege & Marquis 2005; Karban 2011; Wurst & Ohgushi 2015).

To conclude, here we show that community composition as a whole caused a legacy effect, but we do not know whether community structure by itself or a few strongly regulating individual species were mostly responsible for this (Poelman & Kessler 2016). To elucidate the effect of whole communities on individual interactions in the insect-plant interaction web, we need detailed models of species-by-species interactions in the context of a complete community (Poelman 2015; Poelman & Kessler 2016). Also, research on interactions between plants and their biotic environment often focuses on linking plant genotype to phenotype (Barah & Bones 2014), but a better integration of insect communities in this type of research (Stam *et al.* 2014) would allow us to better appreciate how the arthropod community can act as an extended phenotype of a plant (Whitham *et al.* 2003).

Acknowledgements

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Chapter 6

Supplementary material

Interpretation of ordination plots using the biplot rule

The ordination analyses of community compositions in either year resulted in PCA biplots. These graphs show on the first two ordination axes the ordination of response variables, in this case the arthropod species in the community, and explanatory variables, in this case plant seed set. Both are depicted with an arrow from the origin to their ordination on (axis 1, axis 2). PCA graphs can be interpreted using the biplot rule (Šmilauer & Lepš 2014), in which the position of the arrow-tip representing a species is projected with right angles to the (imaginary) line extending through the arrow representing plant seed set. Species whose arrows are long and point in the same direction as the arrow representing seed set are likely to occur on plants that have a large seed set. Contrasting, species with an arrow in the opposite direction of the explanatory variable are more likely to occur on plants that have a small seed set. Finally, if the response and explanatory arrow are perpendicular to each other, then they show no correlation. Furthermore, the shorter the arrow is, the lower the explained variation.

The interpretation between two or more response variables (e.g. between species) is similar; so species whose arrows are long and point in the same direction are likely to occur together on the same plant.

From Principal Component Analysis to Structural Equation Model

The percentage of explained variation by the first PCA axis of herbivore and carnivore community in the first year and second year (Figure 3b and Supplementary Figures 1a, b, c) does not exactly correspond with the percentages given in SEM (Figure 1) even though exactly the same data were used. This is because in the PCA figures presented, the supplementary variable 'Seed set 2013' took up 1 degree of freedom to compose the PCA figures. This supplementary variable was not included in the PCAs that were used as data input for the SEM.

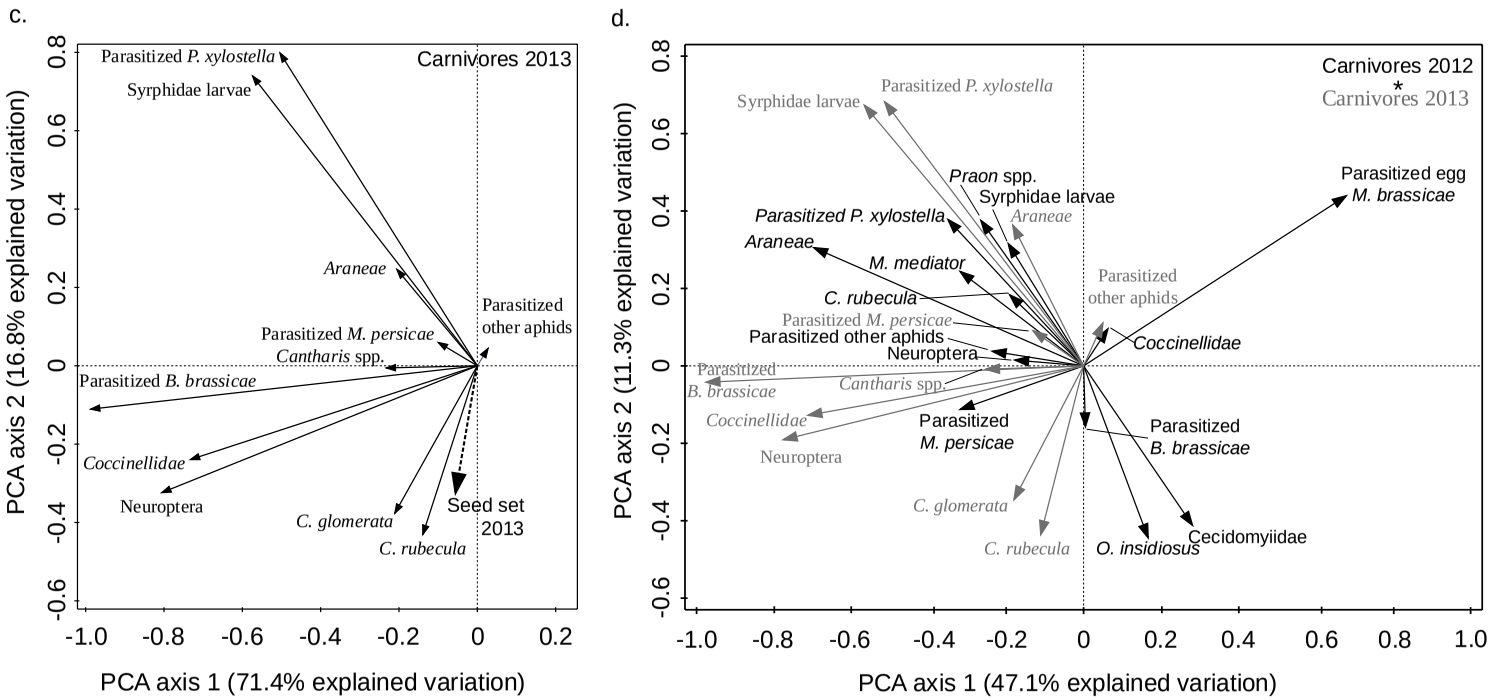
Overlay plot of carnivore community ordination in 2012 and 2013

The overlay plot of the carnivore community ordination in both years as presented in the main text, Figure 3a, is the overlay of Supplementary Figure 1b (carnivores 2012) and Supplementary Figure 1c (carnivores 2013). An overlay is used instead of simply composing a PCA in which both communities are included, because an ordination is determined by all species occurring on a plant (Šmilauer & Lepš 2014). If the ordination would have been made on both communities in a single PCA-plot, as shown in Supplementary Figure 1d, the resulting ordination is different from the ordination of carnivores 2012 (Supplementary Figure 1b) and carnivores 2013 (Supplementary Figure 1c) separately.

In Supplementary Figure 1d however (carnivores 2012 * carnivores 2013 in a single

PCA), note the larger resemblance with the carnivore 2013-arrows of Supplementary Figure 1c (carnivores 2013), than is the case with the carnivore 2012-arrows of Supplementary Figure 1b (carnivores 2012). This is caused by the larger explained variation by PCA of the 2013-carnivore community composition compared to the 2012 carnivore community composition. (Compare % explained variation of first axis of Supplementary figure 1b and 1c.

a.
Herbivores 2013
PCA axis 2 (22.1% explained variation)
PCA axis 1 (31.8% explained variation)
Aleyrodes spp.
A. gamma
Lygus spp.
Gastropoda
P. brassicae
P. xylostella
Leaf miner
B. brassicae
Other Lepidoptera
M. aeneus
Other aphids
A. solstitiale
P. rapae
P. undulata
Thrips
E. forficalis
Philaenus spp.
C. assimilis
E. oleracea
M. brassicae
Seed set 2013
M. persicae
b.
Carnivores 2012
PCA axis 2 (17.2% explained variation)
PCA axis 1 (34.2% explained variation)
Parasitized egg M. brassicae
Parasitized B. brassicae
Coccinellidae
Cecidomyiidae
O. insidiosus
Parasitized other aphids
Seed set 2013
Syrphidae larvae
Parasitized P. xylostella
C. rubecula
M. mediator
Parasitized M. persicae
Praon spp.
Araneae
Neuroptera



Supplementary Figure 1. Principal Component Analysis Ordination biplots of a) Herbivores 2013 with seed set 2013, b) Carnivores 2012 with seed set 2013, c) Carnivores 2013 with seed set 2013 and d) Carnivores 2012 (black arrows) * Carnivores 2013 (grey arrows). Seed set 2013 was not included in the analyses of ordination, but projected on the plots afterwards. % explained variation in community composition by seed set is for a) 7.5%, b) 3.4% and c) 2.3%.

Chapter 7

Plant ontogeny and history of insect attack affect insect community development in a non-additive manner

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Abstract

To cope with unpredictable attack by herbivores, most plant species have inducible defences in which defence levels are enhanced upon actual attack. The phenotypic plasticity in response to an attacker can affect the future assembly of insects on induced plants and may include ecological costs when more destructive herbivores prefer to colonise the induced plant. The costs of herbivore attack and the potential to mount defences also varies over plant ontogeny and results in ontogenetic variation in induced plant responses to herbivory as part of a plant defence strategy. Both the history of insect attacks as well as plant ontogeny are expected to shape plant responses to herbivory, the induced plant phenotype and thereby future insect colonisation. However, it is unknown whether community history and plant ontogeny are additive or not, and if not, whether they act synergistically or antagonistically.

Here, we studied whether feeding by individual insect species or the full history of insect community assembly affects future arthropod colonisation on individual plants and whether these patterns differ over plant ontogeny. In a field experiment with wild perennial *Brassica oleracea* plants, we used a full factorial design in which we manipulated the full history of insect attacks by excluding arthropods by a net for varying periods and assessed the effect of an individual herbivore species on community development by infesting plants with *Pieris rapae* caterpillars at different moments of plant ontogeny. The subsequently arriving arthropod community on the plants was monitored, and we modelled whether the treatments combined resulted in additive or non-additive effects compared to each single treatment, for the future colonisation of arthropods.

We found that community history as well as plant ontogeny shape future arthropod community development. Moreover, the full history of attacks and plant responses to a single herbivore species at different moments of ontogeny were non-additive. For a slight majority of arthropod species the two processes acted synergistically on the preference of species to colonise a plant. However, the direction of colonisation varied widely between species.

These results imply that the strength of plant-mediated interactions among arthropods does not decrease over time, as was predicted from assumptions for induced plant responses underlying the optimal defence theory during plant ontogeny. We discuss the implications of costs of herbivory and of plant defences in plant-insect interactions for future community development.

Keywords

Arthropods, community composition, generalized additive mixed models, herbivory, herbivore-induced plant responses, plant age

Introduction

Plants can be under attack by a wide variety of insects throughout the season (Schmitz *et al.* 2004; Schoonhoven *et al.* 2005; Stam *et al.* 2014). These attacks may be unpredictable and to optimally defend themselves plants need to cope with the pattern by which their community of attackers assembles during their growth (Stamp 2003; Schuman & Baldwin 2016). During the growing season the pool of insect herbivores may build up, and the composition of the insect community may change over time (van Zandt & Agrawal 2004b; Poelman *et al.* 2008a; Poelman *et al.* 2010; Stam *et al.* 2014; Stam *et al.* 2016b). Two elements of a plant defence strategy are considered as adaptations to temporal variation in attack and assembly of insect communities on individual plants: variation in defences depending on plant ontogeny, and variation in defences depending on actual insect attack.

First, plants vary in their investment in defence during ontogeny, because the importance to defend tissues against herbivore attack changes with plant ontogeny (Boege & Marquis 2005; Barton & Koricheva 2010). The optimal defence theory predicts that the more valuable plant parts such as young leaves, flowers and seeds, are better defended than for example older leaf tissue (Stamp 2003; Boege & Marquis 2005; Kessler 2015). Because during plant ontogeny the value of plant parts changes as new tissues are made, plant defences are expected to change over time in a developing plant (Boege & Marquis 2005; Barton & Koricheva 2010; Quintero & Bowers 2011). In addition to this ultimate cause, a proximate cause of variation in plant defence during ontogeny is the capability of a growing plant to defend itself (Boege & Marquis 2005). A young seedling may have fewer resources available for defence than a full-grown plant (Boege & Marquis 2005). Therefore, depending on both the likelihood and costs of insect attack and the available resources during plant ontogeny, the defences to herbivory may show an initial high peak in the sapling stage, but afterwards decrease as the plant gets older (Boege & Marquis 2005; Barton & Koricheva 2010).

Second, to save costs of defence in the absence of herbivores, plants may respond to actual herbivore attack by enhancing their defences (Karban & Baldwin 1997; Schoonhoven *et al.* 2005; Karban 2011; Stam *et al.* 2014). These so-called induced defences also allow plants to respond specifically to the actual type of attacker (de Vos *et al.* 2005; Rodriguez-Saona *et al.* 2010; Agrawal *et al.* 2014). An induced plant response may depend on the feeding guild or even the species of the attacking herbivore (Bidart-Bouzat & Kliebenstein 2011; Ali & Agrawal 2014; Moreira *et al.* 2015). Moreover, combinations of herbivores feeding on a plant induce different plant responses than the sum of responses to each herbivore individually (Kessler & Baldwin 2004; Dicke *et al.* 2009; Soler *et al.* 2012a; Mathur *et al.* 2013; Moreira *et al.* 2015), and the sequence in which they arrive on a plant also influences plant responses (Viswanathan *et al.* 2007; Erb *et al.* 2011; Wang *et al.* 2014; Stam *et al.* 2016b). Even the density of individual insect species affects plant responses

(Křivan & Schmitz 2004; Kroes *et al.* 2015). As a consequence, all changes in the composition of the insect assembly on an individual plant will induce changes in plant phenotypic responses (Utsumi *et al.* 2010; Ohgushi 2016).

Importantly, plant phenotypic variation of ontogenetic nature or induced responses to attack feed back to how the community of insects assembles on the plant individual. Insects select a host plant based on various cues, such as colours or odours emitted by the plant, and the palatability determined by the plant's nutritional value and toxicity due to secondary metabolites (Bukovinszky *et al.* 2008; Dicke *et al.* 2009; Newton *et al.* 2009b; Mithöfer & Boland 2012; Kessler 2015). These cues depend on plant ontogeny as well as induced responses to the cumulative herbivory events. Importantly, an herbivore colonising a plant induces plant responses which will influence the choice of subsequently colonising insects (van Zandt & Agrawal 2004a; Viswanathan *et al.* 2007; Soler *et al.* 2012a; Stam *et al.* 2016a). These subsequent herbivores can induce specific plant responses in their turn, thereby causing a cascade of insect-plant interactions that affects the future insect community dynamics (Ohgushi 2005; Viswanathan *et al.* 2005; Poelman *et al.* 2008a; Utsumi *et al.* 2010; Stam *et al.* 2016b). Thus, insect colonisation and plant responses form a feedback loop that is dynamic over time (Ohgushi 2005; Utsumi *et al.* 2010; Ohgushi 2016). Plant ontogeny further diverges these indirect interactions. A newly arriving herbivore on a young plant, or only a few herbivore attacks on a plant (i.e., a short community history) may evoke a different plant phenotypic response than when a herbivore arrives on an old plant or on a plant with a long community history (Boege & Marquis 2005; Utsumi *et al.* 2010; Utsumi 2015). Therefore, to understand plant adaptations to attack by their insect community, it is important to understand the feedback loop between plant plasticity in defence phenotype and its effect on community assembly. It remains to be identified whether ontogenetic changes in induced responses as well as the history of insect attack have additive or non-additive effects in structuring the assembly of the insect community on an individual plant.

Here, we investigated the importance of arthropod community history and plant ontogeny for the development of the subsequent arthropod community, on an herbivore-induced plant. Specifically, we asked the following questions: I) Does arthropod community history affect colonisation of the plant by new community members? II) Does plant ontogeny affect herbivore-induced plant responses to subsequent arthropod community development? and III) Are community history and plant ontogeny additive or non-additive in shaping arthropod community development?

A common-garden field experiment with wild *Brassica oleracea* plants was set up to answer these questions. We applied variation in community history by excluding the arthropod community for varying time periods with a net, and we inoculated

plants with the leaf-chewing herbivore *Pieris rapae* at different moments of plant ontogeny. Subsequently, the development of the arthropod community was monitored throughout the rest of the season. We used regularized univariate generalized additive mixed models to assess the effects of community history and plant ontogeny on subsequent arthropod colonisation. The models were also used to assess additive or non-additive effects of these factors on colonisation of individual arthropod species. Finally, we evaluated the effects of individual species on the community in more detail. We discuss the results in the context of indirect plant-mediated interactions and the ecological consequences for plant-insect community dynamics.

Materials and methods

Plants and insects

Wild *Brassica oleracea* L. (Brassicaceae) were used for the experiments. Seeds originated from plants growing at Kimmeridge at the Southern coast of England (50°36'N, 2°07'W; Gols *et al.* 2008b). Plants were grown from seeds in a greenhouse in peat soil (Lentse Potgrond No. 4, Lent, the Netherlands), and transplanted into compacted soil cubes about two weeks later. Another two weeks later, seedlings were moved outside under a roof to adapt them to field conditions. In mid-May 2014, when the plants were 5 weeks old and most plants had four true leaves, they were planted in the experimental field site (see below).

To ensure a uniform edge around the field site, two rows of *Brassica nigra* L. (Brassicaceae) plants were planted (see below). Seeds collected from wild plants growing in the vicinity of Wageningen were sown and (trans)planted similar as described for *B. oleracea*.

As an inducing herbivore, the specialist solitary caterpillar *Pieris rapae* L. (Lepidoptera: Pieridae) was used. This is a common herbivore on *Brassica oleracea* and is multivoltine, attacking plants several times during their growth season. The caterpillars originated from a stock culture of the Laboratory of Entomology, Wageningen University, the Netherlands. They were reared on Brussels sprouts plants (*B. oleracea* var. *gemmifera* cv Cyrus) in a climate room (21 ± 1 °C, 50-70% RH, 16L : 8D light cycle).

Field experiment: plant ontogeny and community history

To investigate the relative importance of plant ontogeny and community history in shaping the plant-associated arthropod community on herbivore-induced plants, a field experiment was set up. In a field site in the vicinity of Wageningen University, the Netherlands, 90 plots of 12 plants each were established in an approximately 100 x 50 m field site. The plants were planted at 1 m distance from each other in a 4 x 4 square

per plot, omitting the four central plants to ensure uniform within-plot neighbour conditions. The bare ground within plots was regularly manually weeded. A 3-m-wide strip between plots was sown with a *Poa*/*Lolium* grasses mixture, which was regularly mown during the season. Around the whole field site a double-row edge of *B. nigra* plants was planted, with 1 m between rows and 0.5 m within-row planting distance.

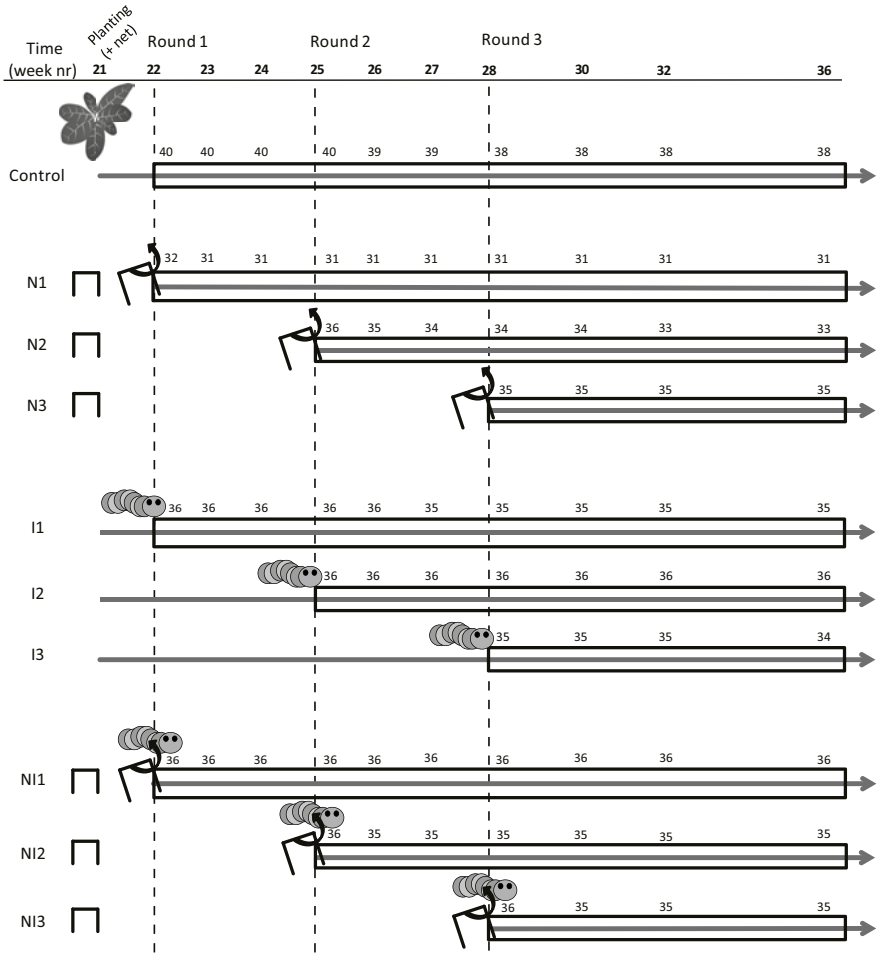


Figure 1: Setup of the field experiment. The horizontal axis represents time, with week number in which a new moment of monitoring started; the different treatments are presented on the vertical axis. The vertical dotted lines indicate the three time points (Rounds) at which the treatments were applied, e.g. removal of a net over the plant (N_i), inoculation with *Pieris rapae* (I_i), and both (NI_i). Control plants did not receive a net, nor *P. rapae* inoculation. Grey arrows indicate the period in which arthropods were present on the plants, while black boxes indicate the period that the arthropod community was monitored at the week numbers indicated. Numbers above each box indicate the total number of plants that were monitored at that moment per treatment (numbers decrease over time when plants died).

In total 10 treatments were defined with four plants per nine plots each (see below and Figure 1). First, in three treatments designed to assess the effect of arthropod community history, the naturally occurring community was excluded from the plants with a net (see below) for a shorter or longer time period, ranging from one, four or seven weeks exclusion. Second, another set of three treatments was used to assess the effect of plant ontogeny in response to herbivory. *Pieris rapae* caterpillars were inoculated on the plants at the same three points in time: one, four or seven weeks after planting (in this study also referred to as early, mid or late-season). Third, the interaction of community history and plant ontogeny was tested with a third set of three treatments in which the exclusion of the community and inoculation with a caterpillar were combined. Immediately after removal of the nets, after a community exclusion period of one, four or seven weeks, *P. rapae* caterpillars were inoculated on those plants. Finally, one set of nine plots was used as control treatment in which the plants were left untouched with neither net, nor caterpillar inoculation. By accident, the plants of one plot in the treatment of community exclusion for one week did not receive nets, and was thus assigned to the control treatment, resulting in 8 and 10 replicates for these treatments respectively.

The day after planting, four plants in each plot were selected to monitor their insect community during the rest of the season. The other plants served as arthropod community 'reservoir'. On the plots that were assigned to exclusion of the arthropod community, these four plants were covered with a gauze net (70x100 cm, mesh size approximately 0.3 x 0.3 mm, cotton-polyester mix (voile)), supported by four Tonkin sticks of 0.9 m long. The top of the sticks and the net were tied together with an elastic band to avoid wind damage to the net, and the bottom of the net was dug into the soil about 5 cm so no insects could enter. No or very few insects had colonised the plants during the first day before placing the nets. The nets and sticks were removed one, four or seven weeks later (Figure 1).

On the plots that were assigned to a treatment with herbivory, all 12 plants received two first-instar *P. rapae* caterpillars one, four or seven weeks after planting. The caterpillars were carefully placed on a fully unfolded horizontal leaf with a fine brush. In plots with a net treatment, the nets were first removed and immediately after that the caterpillars were placed on the plants. The caterpillars were not removed from the plants and caterpillar movement within and between plots was considered negligible due to the bare ground within plots and the grass strip between plots.

On a weekly basis, the arthropod community development throughout the season was monitored on the four assigned plants per plot from the moment of their treatment onwards; control plants were monitored from the first week after planting onwards (Figure 1). In total, the community was monitored ten times during the season, taking 1 - 3 weeks per time point as the season progressed. Both sides of all leaves and stems per plant were carefully inspected for the occurrence of arthropods. All life stages of all insects and other invertebrates such as slugs

and spiders (here commonly referred to as 'arthropods') occurring on the plant were recorded to species level as far as possible. Only fast-flying insects such as adult Lepidoptera and parasitoids were excluded as they could not be assigned to a single plant. The identified insect species and the abbreviations used in this work are listed in Table 1. Per species, all life stages were taken together for the analyses. To assess effects of net coverage on plant growth, the height, diameter and number of leaves per plant were recorded as well at each time point of monitoring.

Table 1: Species as encountered while monitoring wild *B. oleracea* plants during the field experiment, including their taxonomy, feeding type, host specificity, and abundance. [a] Feeding type: Cell content feeder/Leaf chewer/Leaf miner/Phloem feeder/Sap feeder refer to herbivores, in contrast to predators and parasitoids. [b] Specialist/Generalist is based on whether host is within one or more families respectively. [c] Parasitoids of Lepidoptera: cocoons were recorded. [d] Parasitoids of Hemiptera: mummified aphid hosts were recorded. [e] Occ.: the number of non-zero observations out of total 2571 observations during the entire season for all treatments.

Species	Code	Order	Family	Feeding type[a]	Host specificity[b]	Occ.[e]
Autographa gamma	Ag	Lepidoptera	Noctuidae	Leaf chewer	Generalist	85
Mamestra brassicae	Mb	Lepidoptera	Noctuidae	Leaf chewer	Generalist	253
Lacanobia suasa	Ls	Lepidoptera	Noctuidae	Leaf chewer	Generalist	116
Pieris rapae	Pr	Lepidoptera	Pieridae	Leaf chewer	Specialist	1192
Pieris brassicae	Pb	Lepidoptera	Pieridae	Leaf chewer	Specialist	64
Plutella xylostella	Px	Lepidoptera	Yponomeutidae	Leaf chewer	Specialist	1375
Evergestis forficalis	Ef	Lepidoptera	Crambidae	Leaf chewer	Specialist	424
Several other species of *Lepidoptera*	Lo	Lepidoptera	-	Leaf chewer	-	11
Subcoccinella viginti-quatuorpunctata	C24	Coleoptera	Coccinellidae	Leaf chewer	Generalist	13
Phyllotreta undulata	Pu	Coleoptera	Chrysomelidae	Leaf chewer	Specialist	558
Phyllotreta atra	Pa	Coleoptera	Chrysomelidae	Leaf chewer	Specialist	19
Phaedon cochleariae	Pc	Coleoptera	Chrysomelidae	Leaf chewer	Specialist	18
Ceutorhynchus assimilis	Ca	Coleoptera	Curculionidae	Leaf chewer	Specialist	1
Hypera spp	Hy	Coleoptera	Curculionidae	Leaf chewer	?	3
Unknown species of tortoise beetle	Tb	Coleoptera	Chrysomelidae	Leaf chewer	?	15
Several species of *Symphyta* (sawfly) larvae	SyL	Hymenoptera	-	Leaf chewer	-	7
Several species of snails and slugs	SS	- (Class Gastropoda)	-	Leaf chewer	-	285

Meligethes aeneus	M	Coleoptera	Nitidulidae	Pollen feeder	Generalist	2
Brevicoryne brassicae	Bb	Hemiptera	Aphididae	Phloem feeder	Specialist	1559
Myzus persicae	Mp	Hemiptera	Aphididae	Phloem feeder	Specialist	952
Other aphids than *B. brassicae* or *M. persicae*	Ao	Hemiptera	Aphididae	Phloem feeder	-	119
Eurydema oleracea	Eo	Hemiptera	Pentatomidae	Sap feeder	Specialist	1
Aleyrodes spp. (probably *A. proletella*)	W	Hemiptera	Aleyrodidae	Sap feeder	Specialist	225
Lygus spp.	Ly	Hemiptera	Miridae	Sap feeder	-	20
Unknown species of Cicadellidae	Lh	Hemiptera	Cicadellidae	Sap feeder	?	17
Unknown species of thrips	T	Thysanoptera	-	Cell content feeder	-	254
Several (unknown) species of leaf mining insects	Lm	-	-	Leaf miner	-	459
Coccinella spp.	Coc	Coleoptera	Coccinellidae	Predator	Generalist	54
Bembidion quadrimaculatum	Bq	Coleoptera	Carabidae	Predator	?	4
Orius insidiosus	Oi	Hemiptera	Anthocoridae	Predator	Generalist	4
Several species of ants	A	Hymenoptera	Formicidae	Predator	-	25
Several species of Syrphidae larvae	Hf	Diptera	Syrphidae	Predator	Generalist	842
Unknown species of gall midge larvae	GmL	Diptera	Cecidomyiidae	Predator	-	16
Several species of Neuroptera	La	Neuroptera	-	Predator	-	384
Several species of spiders	S	Araneae	-	Predator	-	958
Cotesia rubecula	Cr	Hymenoptera	Braconidae	Parasitoid [c]	Specialist	219
Cotesia glomerata	Cg	Hymenoptera	Braconidae	Parasitoid [c]	Specialist	29
Cotesia lineola	Cl	Hymenoptera	Braconidae	Parasitoid [c]	Specialist	20
Cotesia vestalis	Cv	Hymenoptera	Braconidae	Parasitoid [c]	Specialist	16
Microplitis mediator	Mm	Hymenoptera	Braconidae	Parasitoid [c]	Specialist	21
Praon spp.	Prao	Hymenoptera	Braconidae	Parasitoid [c]	-	80

Unknown parasitoid, probably *Hyposoter* spp.	Hyp	Hymenoptera	Ichneumonidae	Parasitoid [c]	-	5
Species parasitizing *P. xylostella* (likely *Diadegma* spp.)	Ds	Hymenoptera	Ichneumonidae	Parasitoid [c]	-	820
Several species parasitizing *B. brassicae*	BbM	Hymenoptera	-	Parasitoid [d]	-	985
Several species parasitizing *M. persicae*	MpM	Hymenoptera	-	Parasitoid [d]	-	218
Several species parasitizing other aphids than *B. brassicae* or *M. persicae*	AoM	Hymenoptera	-	Parasitoid [d]	-	141
Unknown species of Dermaptera	Ea	Dermaptera	-	-	-	8
Unknown species of mite	Mi	- (Subclass Acari)	-	-	-	16
Other beetles	Bo	Coleoptera	-	-	-	4
Other true bugs	To	Hemiptera	-	-	-	42

Data exploration

In this study, data exploration was performed to reveal any potential errors in the data collection processes as well as factors that could compromise a statistical analysis like unbalanced experimental design, outliers, missing data, too many zero values, and non-linear relationships between the covariates and response variables. Although, the study design was balanced, the resultant dataset was not, because of plants that died during the season and a plot which was not assigned the necessary treatments (Figure 1). However, this did not cause large disparity between the number of samples for each treatment and was therefore ignored. The temporal development of the community on plants appeared to follow a bell-shaped curve which does not cover the entire season (Figure 2). This is a major contributing factor to the large number of zero values in the data. Half of the 50 species which were identified in the experiment were not observed more than 25 times on all plants at all times (Table 1). Only a few outliers in the number of insects and the plant diameter were identified with dot plots (not shown). The insect outliers were not removed because they were a result of investigating all different life stages cumulatively, e.g. eggs and caterpillars. Two outliers in the diameter of the plants were removed because they were ca.10 times larger than the typical diameter and were thought to be caused by mistakes in the data input.

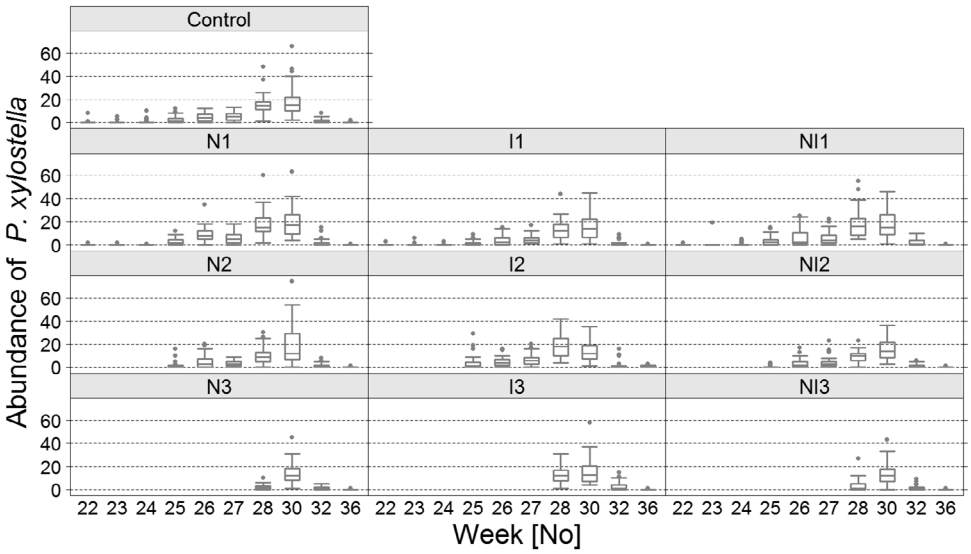


Figure 2: Boxplots of the number of insects at each time point for the caterpillars of *Plutella xylostella* across all plants. It is used to give an impression of the data for abundant species. The rectangles with a line at the middle indicate the 25 %, 50 %, and 75 % quantiles; the whiskers the 5 % and 95 % quantiles; and the dots the outliers.

Estimating effects of net and induction treatments

In order to estimate the effects of community history and plant ontogeny on the future colonization of insect species, the expected number of insects was modelled according to equations 1-3. The effect of placing a net (which studies the effects of community history), a caterpillar (which studies plant ontogenetic changes in herbivore-induced responses), or both (which studies the additive effects of both processes) for all 3 rounds was estimated by constants which reflect a proportionate deviation from the baseline arthropod colonisation on the control plants. Second, to estimate whether community history and plant ontogeny act additively or not in structuring the future insect community, the (non-)additive effect for each round was estimated by a constant which reflects the difference between 1) the estimated effect of placing a net **and** inducing the plants by a caterpillar, and 2) the sum of estimated effects of placing a net **or** inducing the plants by a caterpillar. The baseline colonization was estimated using temporal and auto-regressive O'Sullivan splines. Splines are automatic functions which fit non-linear relationships between covariates and response variables. A temporal spline $f_1(Time\ point)$ was used to capture the probability of occurrence of insects at all time points on all plants during the season. An autoregressive spline $f_2(Insects(Time\ point - 1)$ was used to capture the probability of occurrence of a species when it had already appeared in the previous time step. It captures the immigration (arrival on a plant) and emigration (leaving

the plant or dying) rate of insects. In doing so, it also corrects for the fact that the plants which have been under a net may not have as many insects as the rest of the plants for the first few time steps after the net removal because the community has not yet fully developed. We used O'Sullivan splines as outlined by Wand & Ormerod (2008) and implemented by Zuur & Ieno (2012) with 5 equidistantly spaced knots. The number of knots is deemed sufficient because there are 9 unique time points, and based on the data exploration, autoregressive terms are expected to follow a linear or saturation curve. The correlation of measurements on the same plant was captured by normally distributed constants across all plants. The number of insects that arrived on the plant was corrected for the time since the last measurement had been taken.

$$mu_{i,j,k} = E\,(\#Insects_{Species\ 1,\ time\ point\ j\text{-}1,\ plant\ k}) \tag{1}$$

$$\begin{aligned}\log\,(mu_{i,j,k}) = {} & \varepsilon_k + offset\,(\log(\Delta T)) + f_1(Time\ point\ j) + f_2(\#Insects_{i,\ j\text{-}1,\ k}) \\ & + p_{i,1} * N1_i + p_{i,2} * N2_i + p_{i,3} * N3_i + p_{i,4} * I1_i + p_{i,5} * I2_i + p_{i,6} * I3_i \\ & + p_{i,7} * N1_i * I1_i + p_{i,8} * N2_i * I2_i + p_{i,9} * N3_i * I3_i\end{aligned} \tag{2}$$

$$\varepsilon_k = N\,(p_{1,10},\ \sigma_i) \tag{3}$$

In equations 1-3, E signifies the expected number of insects; N is the normal distribution; ΔT is the time interval since the previous measurement; f's are O'Sullivan splines; ε denotes the normally distributed random errors (intercepts) per plant; σ is the random intercept variance; N1, N2, N3, I1, I2, and I3 have value either 0 or 1, according to the net or induction treatment as indicated in Figure 1. The parameters $p_{i,1..9}$ quantify the (combined) effects for the net and induction treatments for all three rounds separately for each species, and as such, they are the primary goal of this study. The subscripts i, j, k refer to the species, time point and plant a measurement belongs to.

The following paragraph describes in more detail the procedure to get estimates for each species in the community of the effect size and direction of plant ontogeny and community history on the insect colonisation. The expected number of insects, splines, and treatment effects were estimated by performing a regularized generalized regression in Bayesian setting, according to equations 4-8. The number of insects was modelled with a zero-inflated linear negative binomial (NB1, (O'Brien 2011)) distribution to deal with potential over-dispersion. The presence of insects in the first place was modelled with a Bernoulli distribution to deal with the large number of the identified insect species that had a low number of non–zero counts, e.g. see Table 1. The NB1 distribution was implemented to deal with potential over dispersed species. Moreover, we regularized the regression to improve the identifiability of treatment effects for such rare species. To do that, all fixed coefficients were assumed to come from a zero-centered normal distribution. In this approach, the coefficients are shrunk to zero if there is no effect. The stronger

the effects, the lower the shrinkage is, so important parameters remain practically unaffected. This type of analysis produces conservative estimates of the effect sizes in the absence of overwhelming evidence and imposes a minimal bias on strong effects. Moreover, to estimate the evidence of the treatments having an effect in the first place, variable selection was performed with Bernoulli distributed indicator variables following the treatment of O'Hara & Sillanpää (2009), e.g. see eq. 8. The same was done for the splines to validate the effect of time and insects at the previous time step on the observed present number of insects. All parameter priors were weakly (or non) informative. The fixed parameter priors were normal distributions with mean 0 and precision of 0.001. The only exception to that was the probability of occurrence parameter which was modelled with beta distribution with shape and scale parameters of 0.5. All variance parameters were zero truncated t-distributions with 1 degree of freedom, mean of 0, and precision of 0.001.

$$\#Insects_{Species\ 1,\ time\ point\ j\text{-}1,\ plant\ k} \sim NB1\ (mu_{i,j,k} * z_{i,j,k}, size_i) \quad (4)$$

$$z_{i,j,k} \sim Bern(\pi_i) \quad (5)$$

$$p_{i,l} \sim \beta_{i,l} * \gamma_{i,l} \quad (6)$$

$$\beta_{i,l} \sim N(0,\sigma_{i,\ coeff}) \quad (7)$$

$$\gamma_{i,l} \sim Bern(\alpha_i) \quad (8)$$

In equations 4-8, NB1 signifies linear negative binomial distribution (NB1, O'Brien 2011) with dispersion parameter size and mean mu; Bern denotes Bernoulli distribution; z is the Bernoulli distributed random variable; π is the probability of presence or absence of insects; β is the effect parameter if an effect is present; γ is the parameter of effect being present or not; and σ is the standard deviation of the normally distributed effects. The subscripts i,j,k,l refer to the species, time point, measurement, and treatment.

The analysis as described above produced for each of the 50 identified insect species the estimates of effect size and direction of the treatments (community exclusion by a net and caterpillar induction at different moments of plant ontogeny), as well as whether the two processes were additive or not. Next, we performed a meta-analysis to summarize and visualize for all insect species in the community 1) the presence of treatment effects and non-additive effects, and 2) the absolute effect size and direction of the treatments and non-additive effects, e.g. see equations 9-10. A beta-distribution was used to summarize the evidence that a treatment caused an effect. A normal distribution was used to summarize the effect sizes if the treatment caused an effect. In principle, the prior information for the presence and size of an effect is weighted twice, once in the original analysis, and once in the meta-analysis. The meta-analysis pools the estimates which is not reflected in

the individual regression estimates. However, the large number of insects (50) is sufficient to minimize the prior information. Also, the goal of the meta-analysis was to summarize the treatment effects across all species, and not to pool them by imposing a random structure on them.

$$\gamma_{i,l} \sim Beta(\delta_{1,treatment\ l}, \delta_{2,treatment\ l}) \quad (9)$$

$$\beta_{i,l} \sim Normal(0, \sigma_{l,\ coeff}) \quad (10)$$

In equations 9-10, λ signifies the inclusion probability as in equations 6 and 8, β the treatment effect size as in the equations 6 and 7; δ is the parameter for the beta binomial distributed random variable, and σ is the standard deviation of normal distribution for species *i* and treatment *l*.

The validity of the models was evaluated by analysis of the residual patterns and by performing posterior predictive checks. At first, the number of insects was monitored with the more popular zero-inflated negative binomial distribution to account for

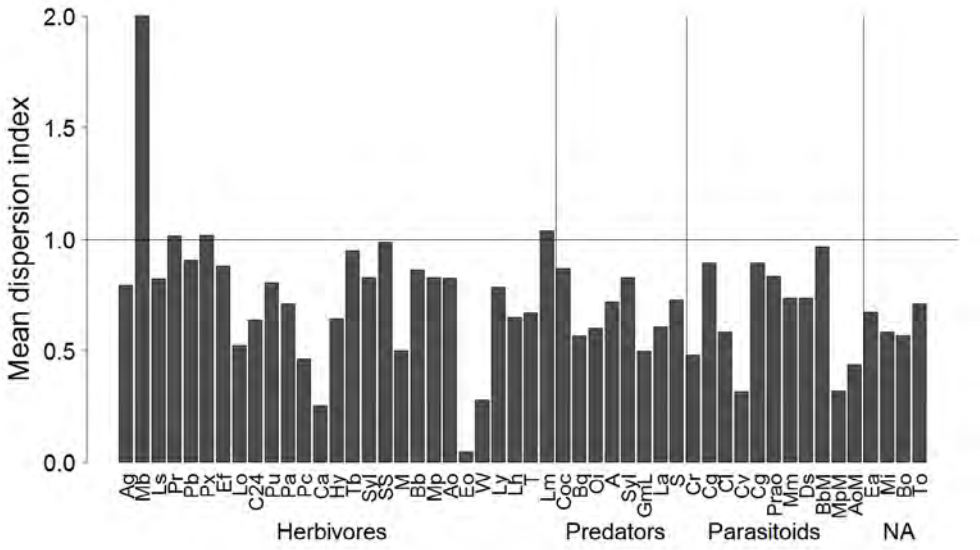


Figure 3: Unadjusted dispersion index for all identified species. The index is defined as the sum of unadjusted squared Pearson residuals. It is theoretically around 1 with values below it indicating under-dispersion and values above it over-dispersion. This relates to the confidence intervals being conservative or optimistic. Most species are under-dispersed because of the low number of non–zero counts and low absolute values. The gregarious species *Mamestra brassicae* (Mb) is over dispersed because of outliers at low fitted values due to sudden egg oviposition in large numbers. See text for more elaborate discussion. Coding of insect species, sorted per feeding type (herbivore / predator / parasitoid / NA) are found in Table 1. NA: feeding type could not be specified.

over-dispersion. However, outliers at low-fitted values created non-linear patterns at high-fitted values. Following the treatment of Ver Hoef & Boveng (2007) we opted for the zero-inflated linear negative binomial distribution which weights high count numbers more than the zero inflated negative binomial distribution. This approach removed the observed non-linear patterns in the residuals (not shown). The dispersion index for virtually all insects was below the theoretical value of 1 which indicates under-dispersion (Figure 3). This means that the confidence intervals of the estimates is conservative. The under-dispersion is the highest for species that do not occur in numbers above 3 to 5 (not shown). The only over-dispersed case is the moth *Mamestra brassicae* which was mainly caused by a few outliers at low-fitted values. This is probably because this gregarious species lays eggs in large batch sizes (often >20 eggs) at once. Still, there were no non-linear patterns in the Pearson residuals against the fitted values, time, and auto-regressive terms (not shown). The residual auto-correlation was minimal and there was no gaping heterogeneity in the residuals among the treatments (not shown).

Estimating effect of nets on plant growth

To test whether placing a net over the plants for a longer or shorter time duration affected plant growth, for example due to a change in microclimate, a generalized (gamma) mixed model was used in Bayesian setting. Height, diameter and number of leaves of individual plants in the first week after a net has been removed were tested for changes in comparison to the control plants which had not been covered with a net (nor been induced with a caterpillar). Thus, the comparison was performed at weeks 22, 25, and 28 which was at the start of Rounds 1, 2, and 3 (Figure 1). The model is presented in equations 11-14 below. The plant characteristics were modelled as gamma distributed variables with a unique shape parameter for each round. The effect of the presence of a net was evaluated by Gibbs variable selection with the help of Bernoulli distributed indicator variables following O'Hara & Sillpanää (2009). The validity of the model was confirmed with residual and posterior checks identical to the ones performed for the insect abundance models as described above.

$$A_{trait\ I,time\ j,plant\ k} = G\left(s, \frac{s}{mu_{i,j,k}}\right) \quad (11)$$

$$\begin{aligned} \log(mu_{i,j,k}) = {} & pp_1 * N0 + pp_2 * N1 \\ & + pp_3 * N0 + pp_4 * N2 \\ & + pp_5 * N0 + pp_6 * N3 \end{aligned} \quad (12)$$

$$pp_i \sim b_{i,l} * \lambda_{i,l} \quad (13)$$

$$\lambda_{i,l} \sim Bern(\alpha_i) \quad (14)$$

In equations 11-14, G signifies the gamma distribution with shape s parameter and mean mu; pp are effect parameters for the no net, N0, and net treatments for all three rounds, N1, N2, and N3; and bb are the effect parameters if there is an effect; λ is the probability of an effect. The indices i, j, and k refer to the trait (height, diameter, number of leaves), time point, and plant a measurement belongs to.

Data, analyses, and software

All the data, analyses, and source codes in R are available at http://tinyurl.com/jty6yvc or can be obtained on request from the corresponding authors. In this work, we used the open source software R, with the supplementary packages lattice, gamm4, and r2jags available on CRAN. The regression models were estimated by the software for Bayesian inference via Gibbs sampling JAGS. The data exploration and additive modelling with O'Sullivan splines was inspired by the work of Zuur & Ieno (2012) and implemented by programming codes from the same source. The codes for the implementation of the negative binomial type 1 distribution in JAGS via the "zeros trick" were also taken from that work (Zuur & Ieno 2012). The models were implemented for all 50 species and three plant properties (height, diameter and number of leaves) with the help of the Amazon EC2 cloud. This was done to spread the computational load among multiple virtual CPUs and speed up the computations from the order of weeks to a day.

Results

Characteristics of the arthropod community

The arthropod community on the *B. oleracea* plants consisted of 50 different species, including herbivores of various feeding types, predators and parasitoids (Table 1). About half of the species were rare, i.e. they did not occur more than 25 times during the whole experiment. The abundance throughout the season of most species shows a bell-shaped curve which does not cover the entire season (e.g. see

for example abundance of *P. xylostella*, Figure 2). This is a major contributing factor to the number of zero's in the data; at many time points at least some species were absent because their peak in abundance was far removed from the time point that was measured.

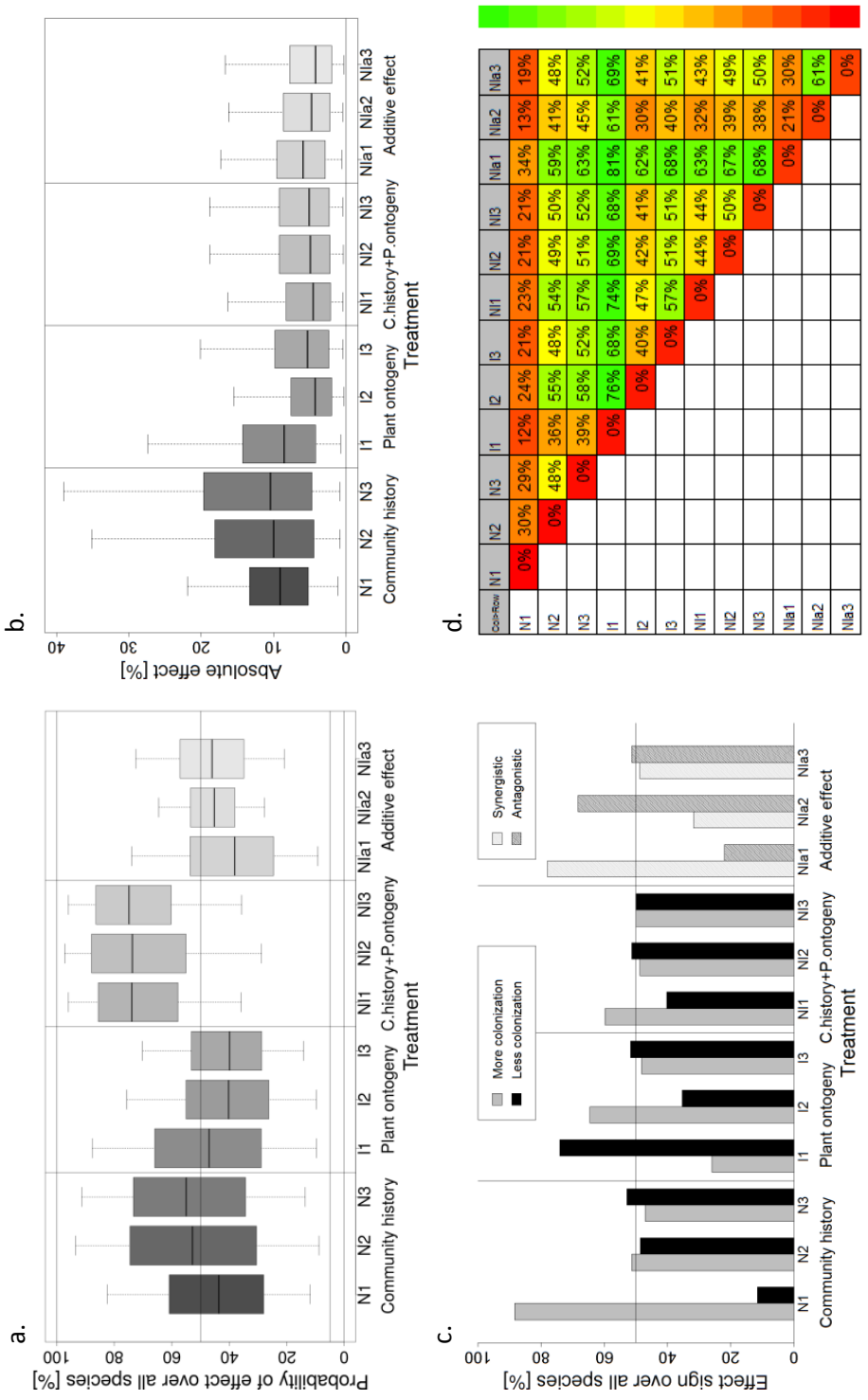
Arthropod community history and plant ontogeny both affect subsequent community development

First of all, we investigated how important the effects of both arthropod community exclusion and herbivore induction during plant ontogeny were for colonisation of all species in the community (Figure 4a). Both community history and plant ontogeny had an important influence on the abundance of insect species in the community (probabilities are >5%; Figure 4a). The median probability of a treatment effect per species on colonisation compared to colonisation on the control plants varied around 50% by both community history (43-55%, N_i) and plant ontogeny (40-48%, I_i; Figure 4a). Although we cannot exclude that our treatments of *P. rapae* inoculation and community exclusion had no effect (e.g. the probability of effects is not 100%; Figure 4a), this is mainly because not all insect species are always present at any given moment (many zero's).

Second, we observed differences between treatments in the absolute size of their effects (Figure 4b), and the direction of effects to arthropod colonisation, e.g., the fraction of species that were colonising the plants more (grey bars) or less (black bars) frequently compared to that on control plants (Figure 4c). This is clear from the overall differences in effect size and direction between treatments (Figure 4d).

The shorter the period during which the plants had experienced a community history (e.g. the longer the arthropods were excluded by a net, N1 - N3), the larger the absolute effect size was of community exclusion on the colonisation of all arthropods (Figure 4b). Also, one week of community absence caused 90% of the insect species to colonise these plants more frequently than control plants (Figure 4c, grey bar N1), while after four or seven weeks of community exclusion, half of the species colonised these plants more (grey bars), and the other half colonised the plants less frequently than control plants (black bars in Figure 4c; see Figure 4d for comparisons between treatments).

Plant response to a single herbivore species (*P. rapae*) at different ontogenetic stages showed a different pattern of effect to future arthropod colonisation than the pattern of effects by community history. The absolute effect size was slightly higher when the caterpillar arrived in the first week, than when *P. rapae* arrived after four or seven weeks (Figure 4b, I_i). However, *P. rapae* inoculation changed the direction of arthropod colonisation when it arrived at different moments of plant ontogeny on a plant with an insect community (I_i; Figure 4c). When *P. rapae* arrived early in the season on younger plants this caused only 28% of the species to colonise these plants more frequently (most species avoided these plants compared


a.
Probability of effect over all species [%]
0
20
40
60
80
100
N1 N2 N3 I1 I2 I3 NI1 NI2 NI3 NIa1 NIa2 NIa3
Community history
Plant ontogeny
C.history+P.ontogeny
Additive effect
Treatment
b.
Absolute effect [%]
0
10
20
30
40
N1 N2 N3 I1 I2 I3 NI1 NI2 NI3 NIa1 NIa2 NIa3
Community history
Plant ontogeny
C.history+P.ontogeny
Additive effect
Treatment
c.
Effect sign over all species [%]
0
20
40
60
80
100
More colonization
Less colonization
Synergistic
Antagonistic
N1 N2 N3 I1 I2 I3 NI1 NI2 NI3 NIa1 NIa2 NIa3
Community history
Plant ontogeny
C.history+P.ontogeny
Additive effect
Treatment
d.
Col-Row N1 N2 N3 I1 I2 I3 NI1 NI2 NI3 NIa1 NIa2 NIa3
N1 0% 30% 29% 12% 24% 21% 23% 21% 21% 34% 13% 19%
N2 0% 48% 36% 55% 48% 54% 49% 50% 59% 41% 48%
N3 0% 39% 58% 52% 57% 51% 52% 63% 45% 52%
I1 0% 76% 68% 74% 69% 68% 81% 61% 69%
I2 0% 40% 47% 42% 41% 62% 30% 41%
I3 0% 57% 51% 51% 68% 40% 51%
NI1 0% 44% 44% 63% 32% 43%
NI2 0% 50% 67% 39% 49%
NI3 0% 68% 38% 50%
NIa1 0% 21% 30%
NIa2 0% 61%
NIa3 0%

◄ **Figure 4**: Effects of community history, plant ontogeny and their (non-)additive effects on arthropod colonisation, and comparisons between treatments and the (non-)additive effects.

a) Probability of presence of effects for all arthropods by each of the treatments (N_i, I_i, NI_i), and their (non-)additive effects (NIa_i). A probability >0.05 for the treatments indicate that the effect of the treatment on arthropod colonisation is very likely present, and a probability >0.05 for the (non-)additive effect indicates that the treatments are non-additive. Quartile boxplots with wishkers representing 95% of the species;

b) Absolute effect size of each of the treatments (N_i, I_i, NI_i) on the colonisation of all arthropod species (the absolute effect size is the summed effect size of both positive effects (more colonisation compared to control) and negative effects (less colonisation compared to control)), and their (non-)additive effects (NIa_i) (an absolute effect >0 is a non-additive effect of plant ontogeny and community history on top of their additive effect). Quartile boxplots with wishkers representing 95% of the species;

c) Fraction of species that colonise the plants more (grey bars) and less frequently (black bars) than on control plants by each of the treatments (N_i, I_i, NI_i), and the direction of their non-additive effects (NIa_i; light grey bars: synergistic effects; dark grey bars: antagonistic effects). Note that the two bars per treatment and time point add up to 100%. Synergistic effects indicate that community history and plant ontogeny change species colonisation (preference or avoidance compared to control plants) in the same direction, while antagonistic effects indicate that community history and plant ontogeny change species colonisation in opposite directions;

d) Comparisons of effect size (see Figure 4b) and direction (negative or positive, see Figure 4c) combined on arthropod colonisation among treatments. E.g., probability that the effects by treatments listed in the column headers are larger than the effects by treatments listed in the row headers (more green). In a similar way for NIa_i, the probability that the (non-)additive effects in the column headers are more synergistic than the (non-)additive effects in the row headers (more green).

Treatment abbreviations: N1, N2, N3: Community exclusion by a net for one, four or seven weeks after planting respectively; I1, I2, I3: Inoculation with *P. rapae* caterpillars at one, four or seven weeks after planting respectively; NI1, NI2, NI3: Combined community exclusion for and *P. rapae* inoculation at one, four or seven weeks after planting respectively; NIa1, NIa2, NIa3: (non-) additive effect of community exclusion and *P. rapae* inoculation as calculated with the formula '$NI_i = N_i + I_i + NIa_i$'.

to control plants), while when *P. rapae* arrived on a plant after four or seven weeks in the field, 65% or 49% of the species preferred to colonise these plants more frequently respectively (grey bars in Figure 4c and see Figure 4d for differences between treatments).

In general, excluding the first community members to arrive on a plant in the first week (N1), or placing *P. rapae* on a younger or older plant which did experience the first arriving community members for one week (I1), showed the most contrasting results to the subsequent insect community development compared to all other

treatments (Figure 4d). Combining community exclusion and herbivore inoculation (NI_i) had intermediate effects, but was not simply additive (see below). However, also in mid- and late-season both community history and herbivore inoculation shaped the later community composition (Figure 4d).

(Non-)Additive effects of community history and plant ontogeny

First, similar to the effects of community history and plant ontogeny, the combination of both factors was important in influencing future arthropod colonisations (probabilities are >5%; Figure 4a). The average probability of an effect of this combined treatment (NI_i) to a community member was 76% (Figure 4a). Looking at the (non-)additive effects of combining the exclusion- and inoculation treatments compared to each of them separate (NIa_i, Figure 4a), shows that on average per species, the probability that the treatments are non-additive is 40-43%. This indicates that non-additive effects of insect community history and the plant response to arrival of an herbivore are very important in shaping the colonisation of insect species on a plant.

Second, the size of effects on arthropod colonisation by the combination of community history and *P. rapae* inoculation followed a different pattern than the effect of either community history or plant ontogeny alone (NI_i; Figure 4b). The median absolute effect size of the combined treatment did not vary much over the season (Figure 4b). However, inoculation of *P. rapae* on plants where arthropods were excluded for 1 week (NI1) caused the majority of species (60%) to arrive more frequently on those plants (Figure 4c), while inoculation after four or seven weeks of community absence induced a nearly equal number of insect species to colonise the plants more and less frequent (Figure 4c, see Figure 4d for the differences between the time points). While this pattern of combined herbivore inoculation and community exclusion was somewhat similar to the pattern of effects of community exclusion alone (see above), the pattern of effects of herbivore inoculation alone was very different (Figure 4c). This results from the non-additive effects of the two treatments (see following paragraph).

At all three time points, the effects of community history and plant ontogenetic variation in responses to herbivory were non-additive when they were combined (95%-whiskers of NIa_i >0 at all three time points; Figure 4a). The absolute size of the non-additive effect, however, slightly decreased when plants grew older (NIa1-3 in Figure 4b).

Whether community history and plant ontogeny enhanced or decreased each other's effects on preference or avoidance of plants by insect species did, however, vary over the season. One week after planting, the effects of community history and plant ontogeny were synergistic for 79% of the species, e.g. community exclusion and *P. rapae* inoculation enhanced each other in their effect on colonisation of arthropod species (light grey bar of NIa1 in Figure 4c). At mid-season, this decreased

to synergism for only 34% of the species, while after seven weeks, 48% of the species experienced synergistic effects of community history and *P. rapae* induction, and the other half of the species experienced antagonistic effects of both treatments (Figure 4c, d). Taken together over the season, however, slightly more than half of the insect species experienced synergistic effects, i.e. they responded in the same way (either avoided or preferred to colonize) to plants with community exclusion and plants with herbivore inoculation (total size of light grey bars NIa_i; Figure 4c).

Effects of community history, plant ontogeny and their interaction on individual arthropod species

Next to the general effect patterns of community history and plant ontogeny on all arthropod species in the community, individual species responded specifically to each of the treatments and at different moments in the season (Figure 5; for data on all species see Supplementary data). For example, the leaf-chewing flea beetle *Phyllotreta undulata* and parasitoids of *Plutella xylostella* colonised plants with an excluded arthropod community (N_i) overall less frequently than control plants exposed to an arthropod community, whereas the aphid *Brevicoryne brassicae*, the caterpillar *P. rapae* and the generalist coccinellid predators colonized these plants without previous insects more frequently. Also two caterpillar species differed in their response to *P. rapae* inoculation at different moments in the season. The generalist *Autographa gamma* colonised *P. rapae*-induced plants (I_i) less frequently than control plants and were neutral when *P. rapae* was the first herbivore when other arthropods had been excluded (NI_i). In contrast, the specialist *P. rapae* seemed to be only mildly positively affected by induction by conspecifics in a full community history (I_i), while they were positively affected when they were the first colonizers on an undamaged plant (NI_i). However, for the latter it should be noted that the effect might seem stronger than it actually is, because the recorded number of *P. rapae* includes the individuals that were experimentally added to the plants.

Also, carnivores were differentially affected by either community history and/or herbivore inoculation at different moments in the season. The colonisation of parasitoids of *P. xylostella* caterpillars was generally decreasing over the season on plants without previous presence of insects, irrespective of *P. rapae* induction (N_i, NI_i). Coccinellidae, however, mostly avoided plants with *P. rapae* induction (I_i, NI_i), but preferred plants on which the insect community had been excluded for one or four weeks (N_i), more or less following the pattern of the aphid *B. brassicae*.

Moreover, whether community history and plant ontogeny acted synergistically (NIa_i effect >0, Figure 5) or antagonistically (NIa_i effect <0, Figure 5) on insect colonisation, also differed per species (Figure 5). For example, the herbivorous beetle *P. undulata* (at the first two time points) and the caterpillar *A. gamma* exhibited synergistic effects in response to both *P. rapae* inoculation and community exclusion, while for example the aphid *B. brassicae* experienced mostly antagonistic

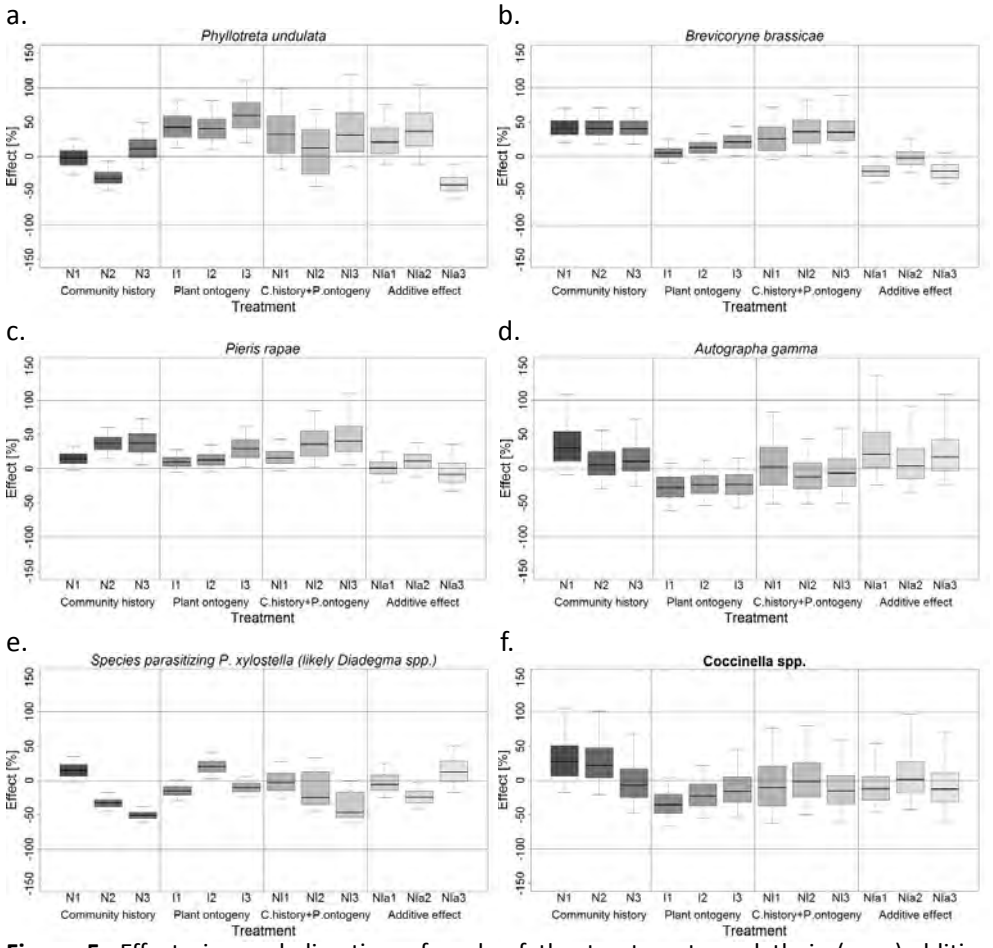


Figure 5: Effect size and direction of each of the treatments and their (non-)additive effects on insect colonisation per species. Six selected abundant species representative of different trophic levels and feeding guilds. a. *Phyllotreta undulata*, a specialist leaf-chewing herbivorous beetle (Coleoptera: Chrysomelidae); b. *Brevicoryne brassicae*: a specialist phloem-feeding herbivorous aphid (Hemiptera: Aphididae); c. *Pieris rapae*, a specialist leaf-chewing herbivorous caterpillar (Lepidoptera: Pieridae); d. *Autographa gamma*, a generalist leaf-chewing herbivorous caterpillar (Lepidoptera: Noctiduidae); e. parasitoids of the specialist caterpillar *Plutella xylostella* (likely *Diadegma spp.*); f. *Coccinellidae* spp., generalist predatory beetles (Coleoptera). Treatment abbreviations: N1, N2, N3: Community exclusion by a net for one, four or seven weeks after planting respectively; I1, I2, I3: Inoculation with *P. rapae* caterpillars at one, four or seven weeks after planting respectively; NI1, NI2, NI3: Combined community exclusion for and *P. rapae* inoculation at one, four or seven weeks after planting respectively; NIa1, NIa2, NIa3: (non-) additive effect of community exclusion and *P. rapae* inoculation as calculated with the formula '$NI_i = N_i + I_i + NIa_i$'. Effect of treatments (N_i, I_i, NI_i) > 0: species is more present compared to presence on control plants; < 0: species is less present compared to presence on control plants; non-additive effects: (NIa_i) >0: synergistic effects; <0: antagonistic effects.

effects when the two treatments were combined. Other insect species, for example *P. rapae* (especially after one week) experienced approximately additive effects; inoculation and exclusion simultaneously affected their presence in the same way as on plants with inoculation and exclusion added up (NIa_i effect = approximately 0). For other insect species, such as parasitoids of *P. xylostella*, and Coccinellidae, the non-additive effects varied over the season (see also Supplementary data for other insect species).

Effects of nets on plant growth

Placing a net over the plants did not have an effect on plant growth (either in height, diameter or number of leaves) after one week (Figure 6a). However, after four weeks growing under a net, plants were slightly taller than control plants; but this difference was absent after seven weeks (Figure 6a, b). Plants grown under a net for seven weeks, however, had a 1.3 times larger diameter (Figure 6c), and approximately 0.6 times fewer leaves than plants that had been growing without a net (Figure 6d).

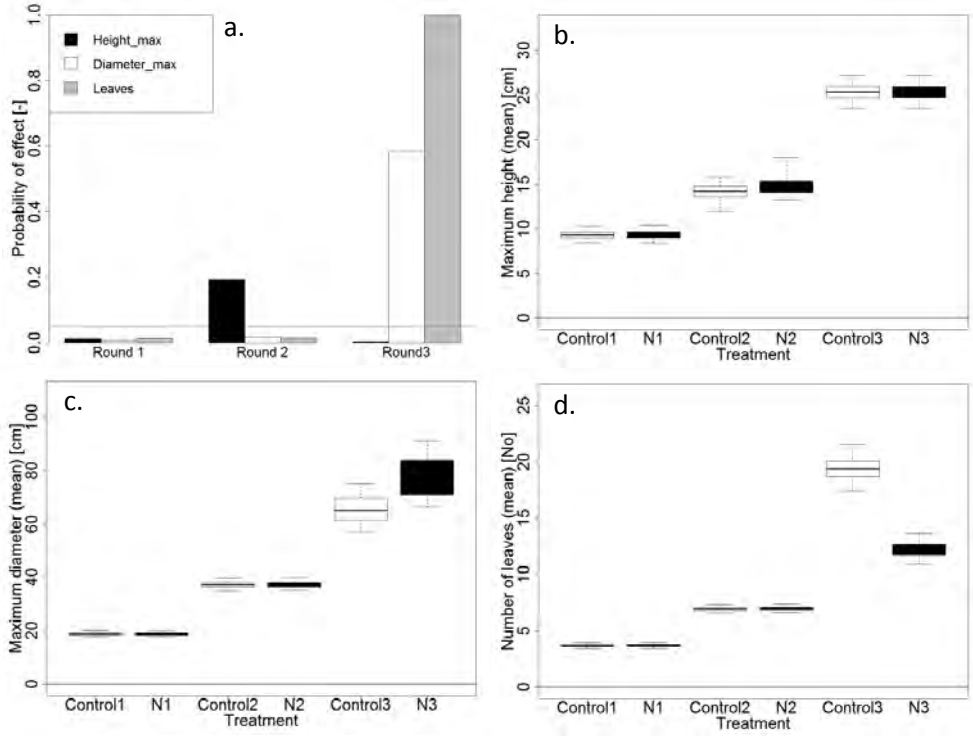


Figure 6. Effects of nets on plant growth. a) Probability of an effect of a net, compared to control plants, on three measurements of plant size: height, diameter and number of leaves. A probability of >0.05 (horizontal line) indicates that an effect of a net on plant size is very likely present. Following panels show estimated means of b) height; c) diameter; and d) number of leaves for control plants and plants covered with a net (N), measured at the three moments at which the net was removed: after one week (round 1), four weeks (round 2) or seven weeks (round 3).

Discussion

A plant phenotype that changes throughout the season can affect the arthropod community composition on the plant (Utsumi *et al.* 2010; Stam *et al.* 2014; Ohgushi 2016). Here, we found that the occurrence of insects on a perennial herbaceous cabbage plant was influenced by community history, plant ontogeny, and the combination of the two at different moments in the season. Furthermore, community history and herbivore induction throughout plant ontogenetic stages are not simply additive, but instead act synergistically or antagonistically in terms of future arthropod colonisation. This contrasts to results from a study on *Plantago lanceolata*, where plant age was more important than the history of insect damage (Quintero & Bowers 2011).

Whether community history and plant ontogenetic changes in herbivore-induced responses enhanced or attenuated each other in terms of the direction of arthropod colonisation, depended both on the moment in the season, and the specific community member. As the season progressed, the colonisation of species was affected increasingly more often in opposite directions by community history and plant ontogeny, but overall, a slight majority of species responded in the same direction to both processes (either preferred both plants with arthropod exclusion and plants with herbivore inoculation, or avoided both) compared to controls. However, individual arthropod species in the community responded very specifically in whether community history or plant ontogeny affected them synergistically or antagonistically.

Thus, all insect interactions until that moment as well as the age of the plant responding to individual herbivores influence the arthropod community composition, and they may enhance each other's effect. This means that even when plants have experienced already many insect attacks, plants may still respond to new attacks by individual herbivores and influence the arthropod community later in the season when they grow older. This has important consequences for the development of the plant-associated community during the course of the growth season.

Although we provide evidence for the non-additive nature of ontogeny and induced plant responses in structuring insect assembly, we cannot exclude the role of other processes in determining insect communities on an individual plant. Also herbivore feeding preferences for different plant ontogenetic stages (Kearsley & Whitham 1989; Barton & Koricheva 2010; Utsumi *et al.* 2013), insect density-mediated effects via direct species interactions (Křivan & Schmitz 2004; Kroes *et al.* 2015) and abiotic effects may have played a role in shaping the future community (Schoonhoven *et al.* 2005; Coolen *et al.* 2016; Davila Olivas *et al.* 2016). For example, growing covered under a net for seven weeks affected plant architecture, but we did not observe any aberrant pattern on the arthropod community, therefore we assume that the proportion of insects affected by plant architecture in this *B. oleracea* system is negligible.

The individual insect species responded very specifically to plant ontogeny and

community history, probably due to large differences in for example their feeding modes and amount and type of damage they inflict to the plant. Unfortunately these differences per species could not be taken into account by the model. The change in abundance of species due to their phenology independent of plant quality was, however, taken into account by the model. By using both a temporal and autoregressive spline, the pattern of abundance over time and the presence of the species at the previous moment of monitoring were respectively captured, and on top of that we could model the effects of community history and plant ontogeny.

Insect community history and plant ontogeny shape community composition over the season

Early-season herbivory can affect the insect community composition on the same plant through plant-mediated indirect interactions between insects (van Zandt & Agrawal 2004b; Poelman *et al.* 2010; Utsumi *et al.* 2010). Here, we show that in addition, plant responses to the community as a whole can affect future insect colonisation as well. When the community was excluded for a certain period, the colonization of subsequently arriving arthropods was affected (while taking into account that some arthropods had been absent beforehand). The community history, i.e. all insects that have been on the plant until that moment, also includes the history of plant responses to these insects, as plants and insects respond to each other in a feedback loop (Ohgushi 2005; Utsumi *et al.* 2010; Ohgushi 2016). Multiple herbivores arriving in various sequences induce different plant responses than simultaneously arriving or single species (Erb *et al.* 2011; Soler *et al.* 2012a; Mathur *et al.* 2013; Wang *et al.* 2014; Coolen *et al.* 2016; Davila Olivas *et al.* 2016). These plant responses affect new colonisers in their choice, and subsequently their performance on the plant, thus shaping the insect community composition (Viswanathan *et al.* 2007; Poelman *et al.* 2008a; Stam *et al.* 2016b). These community members again induce plant responses in a cascading manner (Ohgushi 2005; Utsumi *et al.* 2010). Therefore, the insect community composition at a given moment reflects (in part) all previous interactions of the insects with the plant and *vice versa* (Kostenko *et al.* 2012).

In addition to effects of insect community history, we found that plant responses to an herbivore depended on plant ontogeny. Early, mid or late in the growth season, *P. rapae* inoculation on the plant evoked differences in subsequent arthropod colonisation. The insect colonisation changed from avoidance to preference to an equal frequency of avoidance and preference for the majority of the species as time progressed. This confirms the hypothesis that plant-insect interactions change non-linearly as the plant grows older (Boege & Marquis 2005; Barton & Koricheva 2010; Quintero & Bowers 2011). This may be explained both proximately and ultimately by variation during plant ontogeny in both availability of resources for defence, and

in the need to defend plant tissues (Stamp 2003; Boege & Marquis 2005). However, induced defence responses follow a different ontogenetic pattern in woody versus herbaceous plants (Barton & Koricheva 2010). Woody plants show an increase in resistance from seedling to juvenile stage, and decrease after a peak in the mature stage, as also predicted by Boege & Marquis (2005). Herbaceous plants, however, show a steady decrease in induced chemistry (and an increase in constitutive chemistry; Barton & Koricheva 2010, see also Quintero & Bowers 2011). In our study herbaceous but perennial plants were used from early to late juvenile stage (wild *B. oleracea* only flowers from the second year onwards; see Chapters 4 and 6). During this period, the effect size due to *P. rapae* inoculation did not change much (or slightly increased when *P. rapae* arrived on a previously undamaged plant), which would place this herbaceous perennial plant system under the woody plants in the model of Barton & Koricheva (2010). Perhaps not growth form (herbaceous or woody), but reproductive strategy ((bi)annual or perennial) is a more important determinant of the ontogenetic pattern in herbivore-induced plant responses.

Non-additive effects of community history and plant ontogeny

The processes of community history and plant ontogeny in shaping the future community composition were not simply additive, but instead acted synergistically or antagonistically, depending on the moment and the species affected. Although the two processes, the history of insect attack and plant ontogeny, are not mutually exclusive, a synergy between the two would not seem obvious with respect to plant responsiveness over time. The history of insect-plant interactions would create an increasingly varying plant phenotype over time (Ohgushi 2005; Ohgushi 2016), while on the other hand, plant resistance would steadily increase in premature plants, and decrease in (post)mature plants if the need to defend older tissues decreases (Boege & Marquis 2005). The fact that in the experiments reported here the size of the additive effect did not change over the season and for a majority of the species the two processes acted synergistically, indicates that plant responses to herbivory are still plastic later in the season. This is in contrast to what would be predicted from the assumption that only the most valuable tissues are defended, which changes during plant ontogeny.

Apparently, plants are able to respond not only to early-season herbivores (van Zandt & Agrawal 2004b; Poelman *et al.* 2010; Stam *et al.* 2016b), but also during mid-season to both individual herbivores among the background of the already-present community members (I_i), as well as to the community as a whole (N_i). This indicates that the plant response here was not limited in plasticity of response (e.g. the response was not fixed after damage by the first few attackers; see also (Thaler *et al.* 2002b; Viswanathan *et al.* 2007; Stam *et al.* 2014). Plants could be limited in available resources to respond (Underwood 1998; Karban 2011), which would mean that after continuous attack throughout the season plant response would

reach a certain plateau and cannot respond further, or the response even decreases (Underwood 1998). *Pieris rapae* inoculation on a plant after 7 weeks of exposure to a diverse insect community still evoked differences in subsequent arthropod colonisation, indicating that here such a plateau was not (yet) reached.

Here, *P. rapae* as an individual herbivore induced similar sizes of effects to future insect colonisation as did the community as a whole. However, plants may not be responding to every herbivore species in the community, but only to species that are posing the largest cost of damage (Kessler & Baldwin 2004), or those that, through interactions with other community members, play a keystone role in the community composition (Poelman & Kessler 2016).

Conclusions and future perspectives

Here, we identified that plant ontogeny and community history interact in shaping the future insect community on a plant. Therefore, a cascade of plant-mediated interactions between insects (Ohgushi 2005; Utsumi *et al.* 2010; Ohgushi 2016) can continue throughout the development of a plant, but the interactions may be modified over time by the plant's developmental stage. So, in addition to plant phenotypic plasticity (Ohgushi 2016), also plant developmental stage should be incorporated when we fully want to understand plant-insect community dynamics over time.

We did not find a decrease in responsiveness to herbivory when the plant gets older, as was predicted by Boege & Marquis (2005), although in our study the mature plant stage was not reached yet. A continued study over several years of this perennial plant-insect system might have shown a decrease in responsiveness to herbivory, although it was found in this system that plant-mediated effects of the insect community lasted across years and still affected the later community dynamics in the flowering phase (Chapter 6). This does not indicate that plant responsiveness decreases over time. However, not in all long-term studies ontogeny effects are taken into account, while the ontogenetic stage of a plant can have a large impact on the outcome of plant-insect interactions (Wurst & Ohgushi 2015).

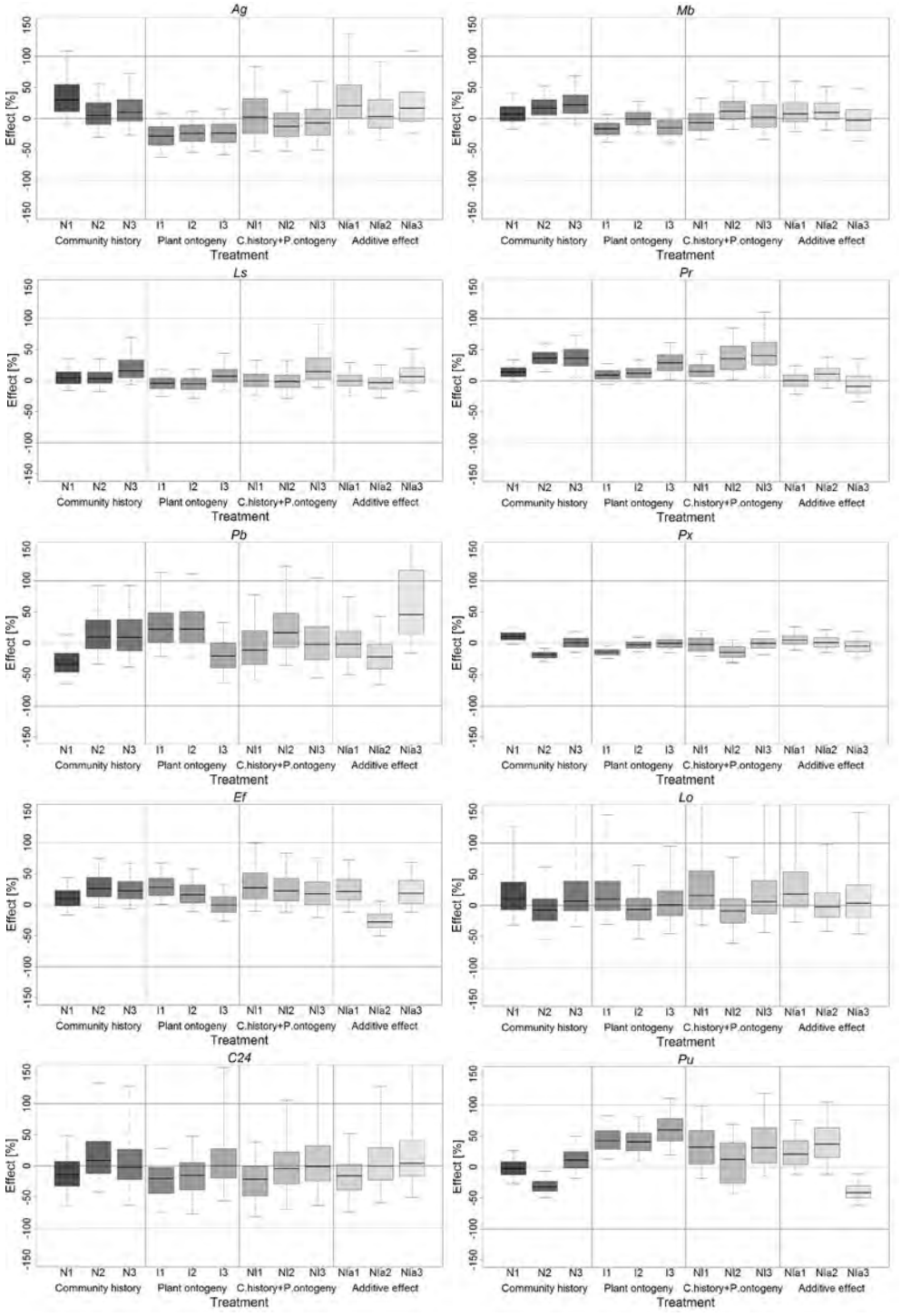
The non-additive effects of plant ontogeny and community history in shaping the subsequent community dynamics as identified here, have consequences for interpreting the adaptability of phenotypic plasticity in herbivore-induced plant responses. In a system where insect attacks are unpredictable and the costs of attacks variable due to plant ontogeny, such non-additive effects may indeed be adaptive from the plant's perspective. However, we need to know more about both the predictability of insect community dynamics (Poelman & Kessler 2016) and changing costs and benefits of attacks during ontogeny (Boege & Marquis 2005; Barton & Koricheva 2010) to fully understand the adaptability of plant phenotypic plasticity.

Acknowledgements

We thank Rieta Gols for providing the *B. oleracea* seeds and the insect rearing team of the Laboratory of Entomology, especially Léon Westerd, Joop Woelke, and André Gidding, for kindly providing *P. rapae* caterpillars. Thank you to the people of Unifarm for helping with planting and maintaining the field site. This study was supported by a TOP GO grant (no. 854.10.010 to Marcel Dicke) from The Netherlands Organization for Scientific Research (NWO).

Chapter 7

Supplementary material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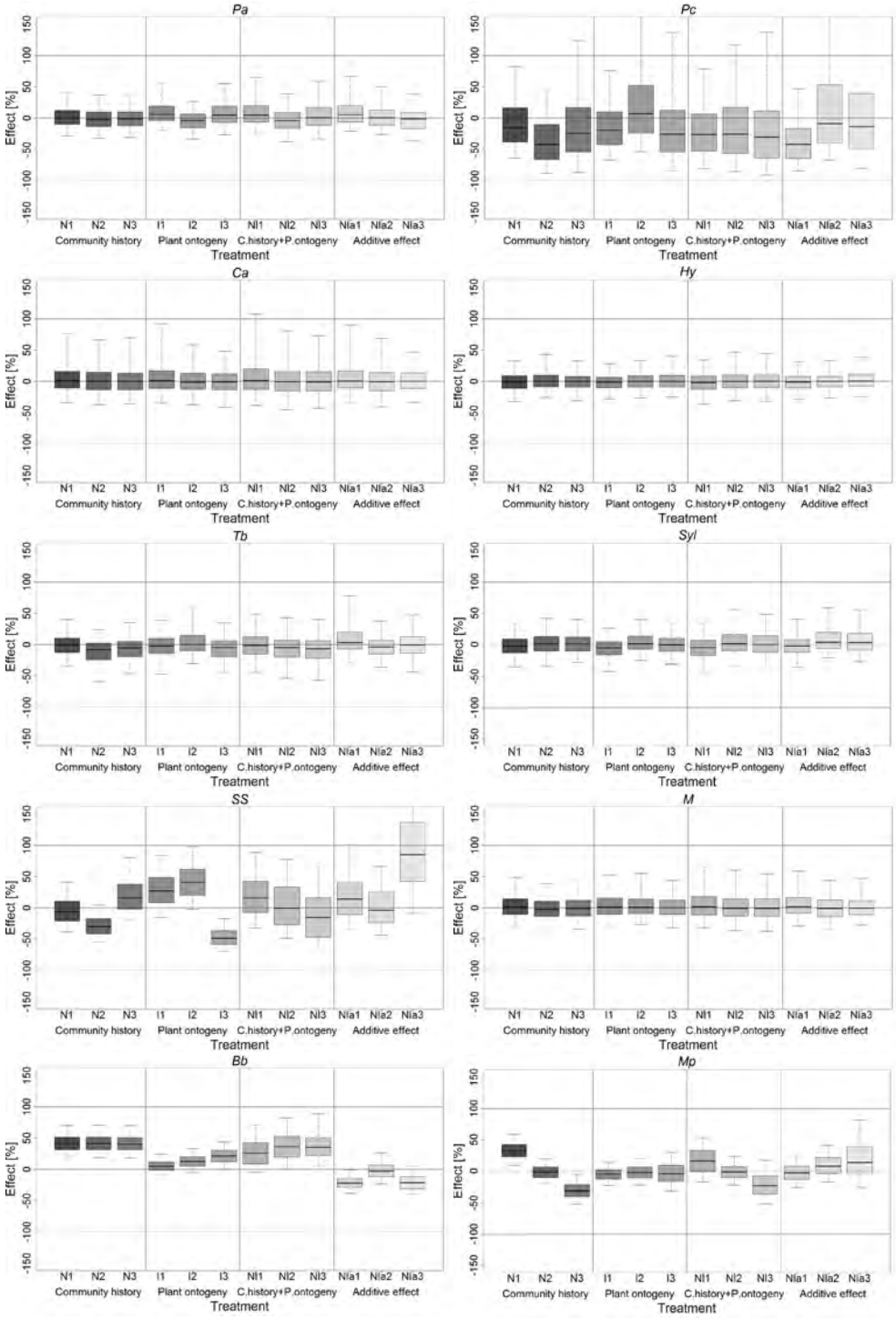
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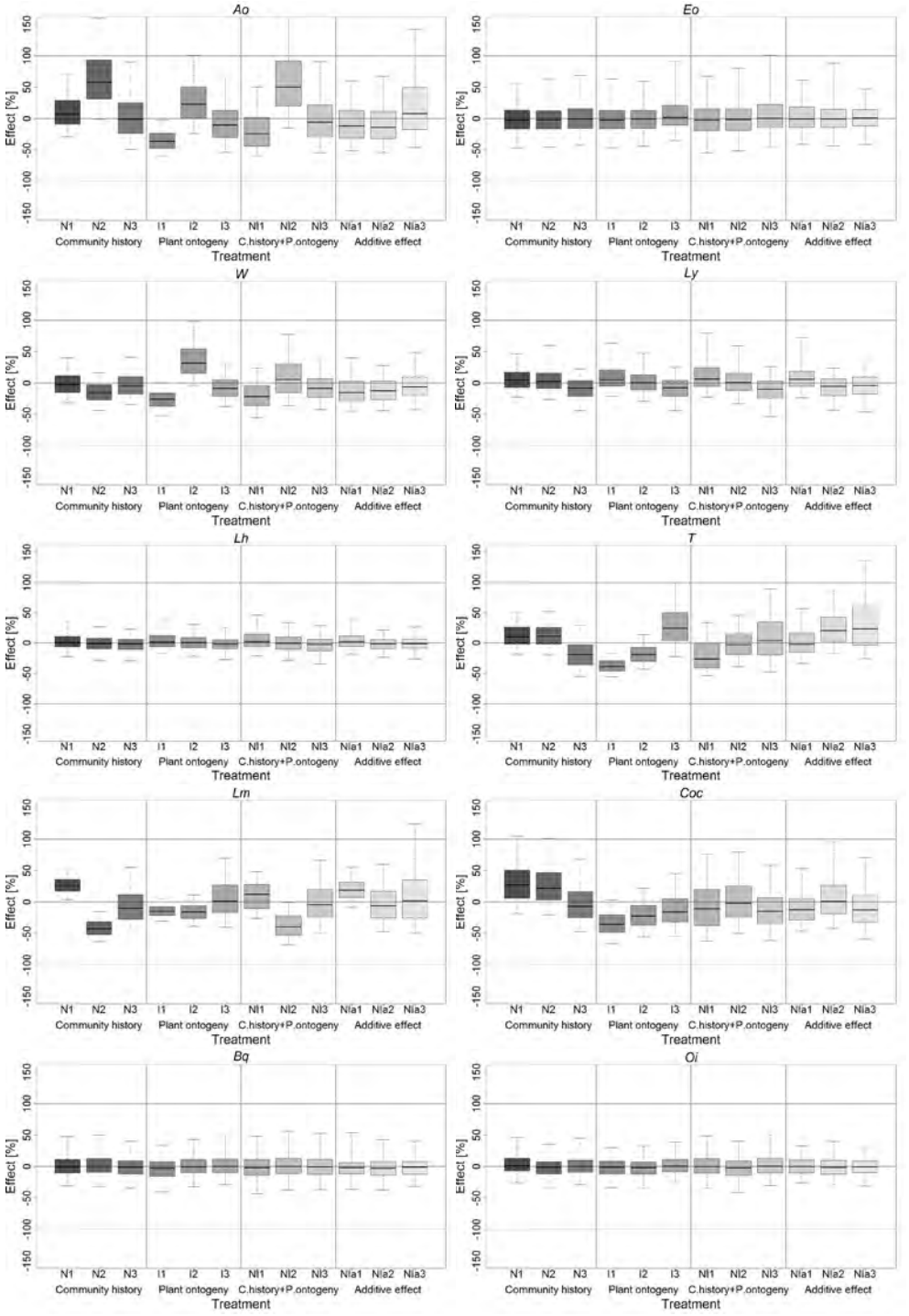
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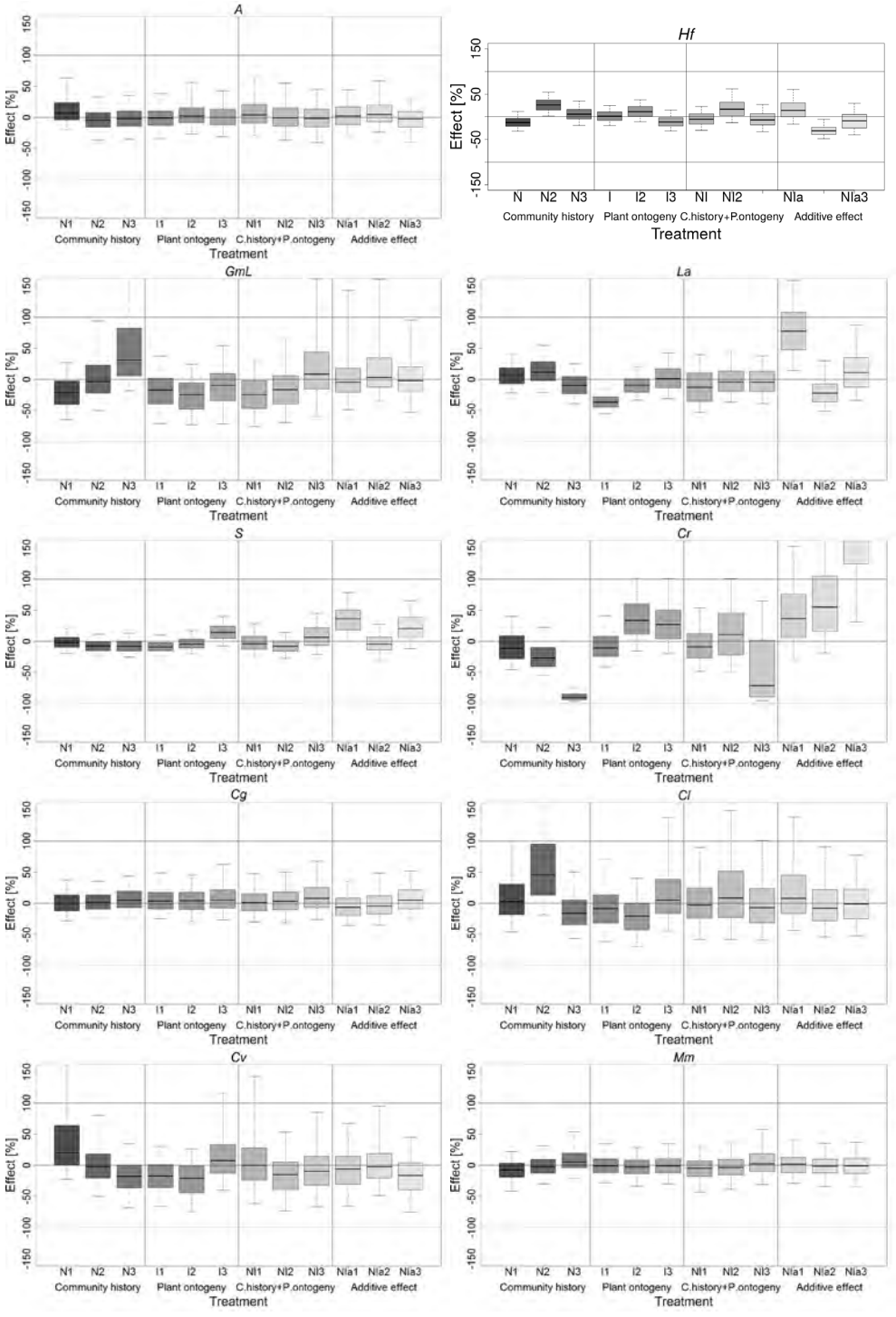
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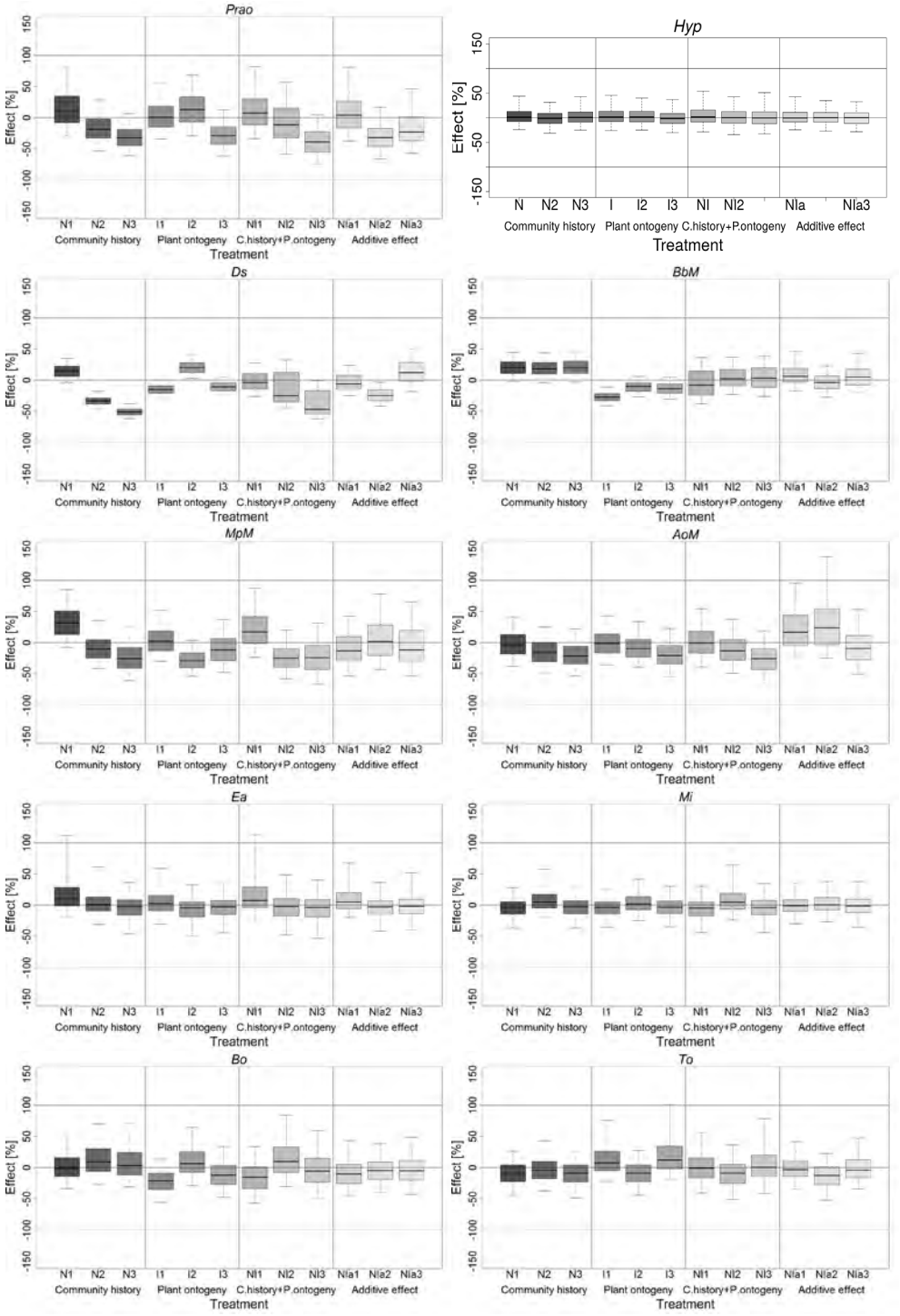
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Plant ontogeny
C.history+P.ontogeny
Additive effect
Treatment

◄ **Supplementary Figure**: Effect size and direction of each of the treatments and their (non-)additive effects on insect colonisation for each of the 50 species observed while monitoring wild *B. oleracea* plants during the field experiment as described in the main text. For species names per code, see Table 1 in main text.

Treatment abbreviations: N1, N2, N3: Community exclusion by a net for one, four or seven weeks after planting respectively; I1, I2, I3: Inoculation with *P. rapae* caterpillars at one, four or seven weeks after planting respectively; NI1, NI2, NI3: Combined community exclusion for and *P. rapae* inoculation at one, four or seven weeks after planting respectively; NIa1, NIa2, NIa3: (non-) additive effect of community exclusion and *P. rapae* inoculation as calculated with the formula '$NI_i = N_i + I_i + NIa_i$'. Effect of treatments (N_i, I_i, NI_i) > 0: species is more present compared to presence on control plants; < 0: species is less present compared to presence on control plants; non-additive effects: (NIa_i) >0: synergistic effects; <0: antagonistic effects.

Chapter 8

General discussion

Introduction

Plants that are fed upon by herbivorous insects can respond with a change in phenotype (Haukioja *et al.* 1985; Karban & Baldwin 1997; Schoonhoven *et al.* 2005; Ohgushi 2016). This changed phenotype is a resistance response when it negatively affects the attacking insect, or future attacking insects (Karban & Baldwin 1997; Poelman 2015). However, also positive effects of herbivore-induced plant responses to herbivore performance occur, for example when an insect is adapted to use plant secondary metabolites as a feeding stimulant (van Loon *et al.* 2002; Schoonhoven *et al.* 2005). Via these plant phenotypic changes, insects can interact with each other and influence the behaviour of other insect individuals (Agrawal 2000; Rodriguez-Saona *et al.* 2005; Zhang *et al.* 2009; Li *et al.* 2014) as well as the dynamics of whole insect communities (van Zandt & Agrawal 2004b; Viswanathan *et al.* 2005; Poelman *et al.* 2008a; Poelman *et al.* 2010).

The induced phenotype of a plant varies with the type of feeding mode or even species of the feeding insects (de Vos *et al.* 2005; Kessler & Halitschke 2007; Bidart-Bouzat & Kliebenstein 2011; Ali & Agrawal 2014). Most importantly, if more than one species eat together from a plant, the resulting plant response is different from the sum of responses to each of the herbivores alone (Rodriguez-Saona *et al.* 2010; Soler *et al.* 2012a; Mathur *et al.* 2013; Davila Olivas *et al.* 2016). We have, however, limited knowledge on what this means for other insects subsequently sharing the same plant, their interactions in the insect community, and how this feeds back to the plant.

In this thesis, I have studied plant interactions with *multiple* herbivores, and especially which consequences dual herbivory has for subsequently arriving insects that have to deal with a dual-herbivore induced plant phenotype. In addition to the identity of different attacking herbivore species, also their timing of arrival on a plant and the identity of the plant population may affect how subsequent insects respond in terms of preference, performance, and community assembly. Overall, the following findings from this thesis contribute to the knowledge on insect - plant interactions in a community context.

First of all, in accordance with previous studies on the specificity of induced plant responses by insects from different feeding guilds (van Zandt & Agrawal 2004a; de Vos *et al.* 2005; Ali & Agrawal 2014), also in this study system we found that the caterpillars of the diamondback moth, *Plutella xylostella*, induced different plant responses than did the aphid *Brevicoryne brassicae*. This led to different behaviours, performance and community dynamics of insects that subsequently arrived and fed on the plant induced by either aphids or caterpillars (Chapters 2, 3, 4, 5). In a field setting the aphid *B. brassicae* had a stronger effect on the subsequent community composition than the caterpillar *P. xylostella* did (Chapter 4), while both insects had similar effect sizes on an individual, subsequently arriving, herbivore under

greenhouse conditions (Chapters 3 and 5). The absence of differences in induced responses by two single herbivore species in the laboratory could perhaps be due to the higher densities that are reached in the field by *B. brassicae* compared to densities of *P. xylostella*, and insect density has been shown to affect plant responses (Křivan & Schmitz 2004; Kroes *et al.* 2015).

Second, plant responses to the two herbivores *P. xylostella* and *B. brassicae* generally followed predictions from the literature on differential induction of phytohormonal pathways by insects from different feeding guilds (Chapter 5): leaf-chewing caterpillars induce the jasmonic acid (JA) pathway, while phloem-feeding aphids induce the salicylic acid (SA) pathway (Thaler *et al.* 2002b; de Vos *et al.* 2005; Pieterse *et al.* 2009). However, we reported nuances in this general pattern depending among others on herbivory duration and plant population, and we observed that the caterpillar *P. xylostella* can also induce SA (Chapter 5). Previous feeding by the caterpillar *P. xylostella* had a negative effect on the performance of a subsequently arriving caterpillar, *Mamestra brassicae* (Chapter 3). This was expected, because herbivory by a leaf-chewing caterpillar is assumed to trigger plant defences that negatively affect other leaf chewers (Karban & Baldwin 1997; Schoonhoven *et al.* 2005; Kaplan & Denno 2007; Erb *et al.* 2012; Underwood 2012; Stam *et al.* 2014). However, we did not observe a facilitation effect of a species from one feeding guild to the performance of a species from another feeding guild, through an alleviation of plant resistance responses by the former insect (Kessler & Baldwin 2004; Zhang *et al.* 2009; Soler *et al.* 2012a).

Plant-mediated interactions with multiple herbivory

An important conclusion throughout this thesis is that dual herbivory induces different, non-additive plant responses and they result in different consequences for subsequently arriving insects and insect communities than the effects of each of the herbivores alone (Chapters 2, 3, 4 and 5). Multiple herbivory thus is fundamentally different from herbivory by a single herbivore, which is important to realize as plants are hardly ever fed upon by a single insect species only. Plants have evolved under this multi-herbivore pressure instead of herbivory by single species only (Schoonhoven *et al.* 2005; Stam *et al.* 2014; Ohgushi 2016; Poelman & Kessler 2016).

A consequence of more than one herbivore arriving on a plant, is that the timing at which they arrive (e.g. simultaneous versus sequential) may vary. Simultaneous arrival of two herbivore species had different effects on plant responses and responses of subsequent herbivores or other community members, than when herbivores arrive in sequences (Chapters 3 and 4; Viswanathan *et al.* 2007; Erb *et al.* 2011; Wang *et al.* 2014). For example, in my field studies the development of the parasitoid community differed between plants on which caterpillars and aphids were inoculated simultaneously, or when caterpillars were inoculated a week later

than the aphids (Chapter 4). Furthermore, the duration of the time lag in arrival between sequentially arriving herbivores may vary (Karban 2011; Underwood 2012), and also this may affect subsequent feeders (Chapters 3 and 5). For example, the caterpillar *M. brassicae* grew better when feeding on plants on which the second herbivore arrived increasingly later than the first herbivore (Chapter 3).

Finally, with two herbivore species arriving one after another on a plant, not only the timing, but also the order of arrival can vary (e.g. caterpillars arriving first or aphids arriving first). Also this had a pronounced effect on future herbivores feeding on the same plant, although the effect was observed more clearly in a field setting where the whole arthropod community was involved (Chapter 4), than for a single responding herbivore under greenhouse conditions (Chapter 3). Effects of order of herbivore arrival were previously found in aboveground-belowground systems in both field and greenhouse experiments (Erb *et al.* 2011; Wang *et al.* 2014).

Interestingly, variation in the timing between the arrival of two herbivore species can have equal or even stronger effects than variation in their order of arrival, for a next herbivore feeding on the same plant (Chapter 3).

Long-term consequences to community dynamics, and variation in responses between plant populations

Plant resistance responses to herbivores can have effects that last much longer than the couple of days or weeks that those herbivores are present on the plant (Kostenko *et al.* 2012; Wurst & Ohgushi 2015; Chapters 6 and 7). A single herbivore within the large insect community that occurs on a plant could still evoke a change in the colonisation of future community members on that plant (Chapter 7). Apparently, plants can respond to individual community members while being in interaction with several others. Moreover, not only a single herbivore, but also the community composition as a whole can have long-lasting effects on both insects and plants. The carnivore community composition affected next year's carnivore community composition, and the herbivore community affected the fitness of the plant in the following season (Chapter 6).

Plant fitness, measured here as the number of seeds that were produced at the end of the first flowering season, was only marginally affected by herbivory of one or two species early in the season (Chapters 4 and 6), but an herbivore community as a whole did affect the plant fitness, even a year later (Chapter 6).

In addition, closely related plant species that have nonetheless experienced different selection pressures from the insect community with which they interact, likely also have evolved different responses to an herbivore (Ali & Agrawal 2014). Even within a plant species, populations may show different herbivore-induced responses (Gols *et al.* 2008b), and here we observed that three wild cabbage populations differ in their responses to caterpillar and/or aphid feeding (Chapters 4 and 5). Induction with

herbivores early in the season resulted in a different insect community composition between two plant populations. This indicates that the induced responses may have differed, either in intensity and/or direction, between the populations, on top of population-specific constitutive phenotypes (Chapter 4). Therefore, within a species, plants may have evolved different strategies of resistance responses to multiple herbivory.

In conclusion, in this thesis I have shown that the phenotype of a dual-herbivore-induced plant forms a different food source or habitat to deal with for a next insect, than the phenotype of a single-herbivore-induced plant. Importantly, this dual-induced plant phenotype subsequently shapes community dynamics through interactions between future arriving arthropods and the plant. In the following sections, I will discuss how the timing of multiple herbivory influences the insect community dynamics mediated by the plant, and the consequences that this has for insect-plant interaction dynamics. Thereafter, I will discuss the consequences of a dynamic community for both the plant and the insect community, and I will conclude with an outlook to future research.

Timing of plant-mediated interactions between multiple herbivores

Timing is an important and recurrent aspect in the study of plant responses induced by insects (Gomez *et al.* 2010; Karban 2011; Underwood 2012). Researchers have studied for example the timing of herbivore arrivals on a plant (e.g. simultaneous versus subsequent arrival: Kessler & Baldwin 2004; Voelckel & Baldwin 2004; Miller-Pierce & Preisser 2012; Soler *et al.* 2012a; Moreira *et al.* 2015); the time lag of plant response after the onset of herbivory (Gomez *et al.* 2010; Mouttet *et al.* 2013) and the decay lag after ceasing of herbivory (Underwood 1998; Voelckel & Baldwin 2004; Gomez *et al.* 2010; Underwood 2012). In addition it has been studied in detail, but often only for a short time period, how plant responses to attackers change over time (Ehlting *et al.* 2008; Mai *et al.* 2014) and how insect responses to plant defences change over time (Underwood 1998; Gomez *et al.* 2010; Mathur *et al.* 2013). In many of these studies on the timing of insect-plant interactions, plant physiology and its kinetics play a central role to explain the observed variation in plant and insect responses to the aspects of timing (e.g. Kessler & Baldwin 2004; Voelckel & Baldwin 2004; Ehlting *et al.* 2008; Mai *et al.* 2014). Despite these studies, we still do not know much about the kinetics of plant physiological responses to herbivory, and the consequences for preference and performance of subsequent insects or the dynamics of insect communities, especially in the long term.

Throughout this thesis, I have studied the consequences for insect behaviour and insect community dynamics of various aspects of timing of multiple-insect - plant interactions (Figure 1). Although we know only a little about the underlying

mechanisms which cause variation in insect-plant interactions due to timing, the (proximate) causes for this variation likely have to be sought in the physiology of the plant (Schuman & Baldwin 2016) and in the feeding behaviours and phenology of all, or the most important, insects in the plant-associated community (Gols *et al.* 2011; Utsumi 2015; Poelman & Kessler 2016). Moreover, placing these mechanisms in an insect-community framework is a major challenge in ecology (Poelman & Kessler 2016). In the following paragraphs I will discuss which mechanisms and consequences of timing may be important for I) the general course of multiple-herbivore-induced plant responses during the time frame of minutes to months to years (Figure 1); II) the effects that order of arrival and time between arrivals of two herbivores from different feeding guilds have on plant physiological responses (Figure 2); and III) how plants prioritize their responses to multiple herbivores from different feeding guilds throughout the season (Figure 3).

Time course of plant responses to multiple herbivory over minutes to months to years

Processes that can occur in very short time frames, like the initial plant response to herbivory within minutes or a day after attack (Voelckel & Baldwin 2004; Ehlting *et al.* 2008; Mai *et al.* 2014; Schuman & Baldwin 2016), can have consequences for processes on a much longer time scale, for example the assembly of a plant-associated community months or even years later (van Zandt & Agrawal 2004b; Viswanathan *et al.* 2007; Poelman *et al.* 2008a; Nuttle *et al.* 2011; Wurst & Ohgushi 2015). Such short- and long-term processes may be connected in several ways (Figure 1).

First, plants may show a time lag between the start of herbivory and a response that could influence the herbivore (Underwood 1998; Karban 2011; Vos *et al.* 2013a; Figure 1). Even though changes in gene regulation of the plant after herbivory have been shown to occur within hours (Ehlting *et al.* 2008; Schuman & Baldwin 2016), and the upregulation of phytohormonal signals can occur within hours to a few days (Halitschke *et al.* 2001; Ziegler *et al.* 2001; Glauser *et al.* 2008; Mai *et al.* 2014), the consequences of these gene- and hormonal changes for, for example, the production of secondary metabolites may take a few days to a few weeks (Kuśnierczyk *et al.* 2008; Erb *et al.* 2012; Mathur *et al.* 2013; Barah & Bones 2014; Wang *et al.* 2015). Only the latter forms a 'meaningful' phenotype (or 'appropriate response'; Erb *et al.* 2012) in perspective of an herbivore, e.g. a change in the plant phenotype that can affect an herbivore in its behaviour, growth or survival (Schoonhoven *et al.* 2005; Poelman 2015). Still, insect herbivores can show measurable responses to an induced plant phenotype after several hours or days, apparently already responding to herbivore-induced plant changes that can be measured earlier, such as volatile production (Underwood 1998; Dicke *et al.* 2009; Underwood 2012; Mouttet *et al.* 2013).

Second, once a plant phenotype is induced within a few hours or days by an herbivore

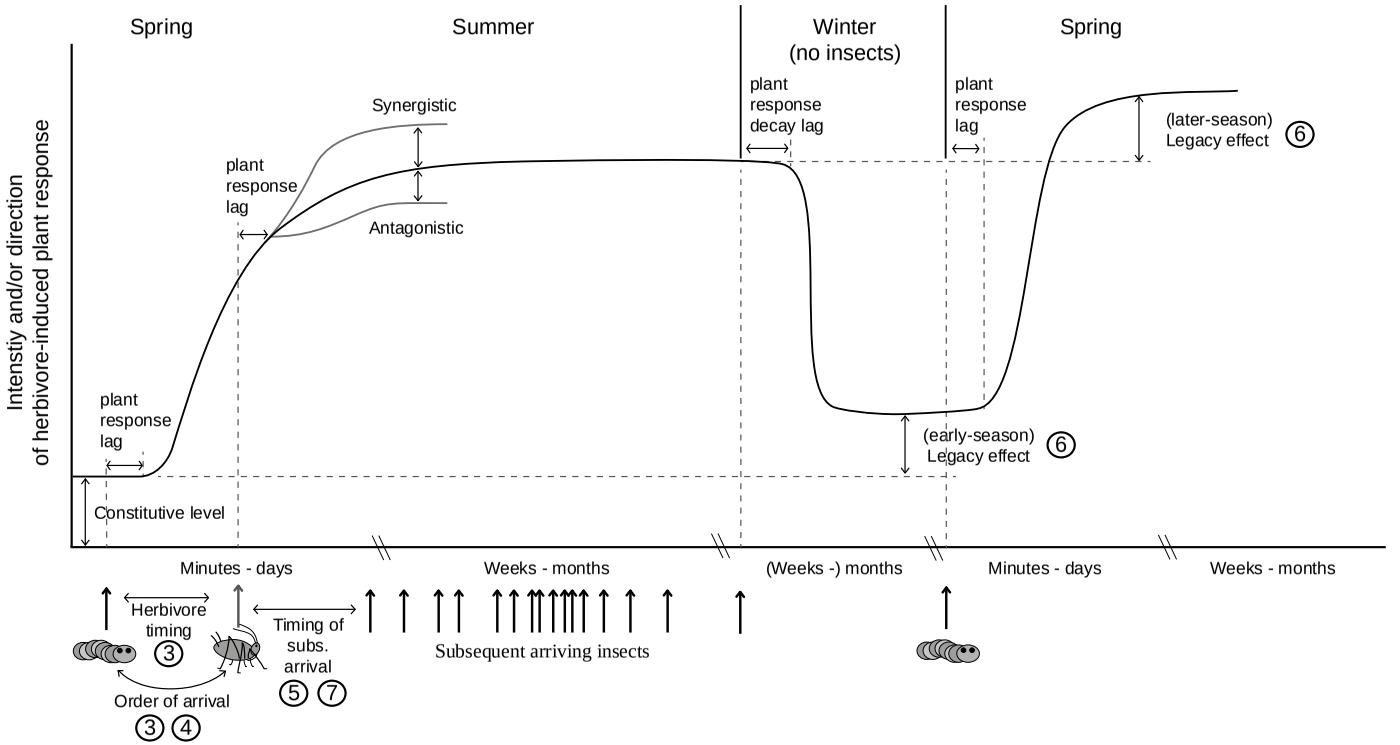

Figure 1. Time course of plant responses to multiple herbivory from minutes to months to years. The theoretical intensity and/or direction of herbivore-induced plant responses is depicted over time; see text for details. The response intensity and/or direction should be interpreted as a certain change in plant phenotype as a whole, not as a quantitative measure of a single plant trait. Vertical arrows represent insects (herbivores and other trophic levels) arriving on and interacting with a given perennial plant. Numbers in circles indicate the Chapters of this thesis in which the different time-related aspects of herbivore-induced plant responses are studied. Note that the time scale changes from minutes - days to weeks - months and back, to compare processes that occur at different time scales over the course of a year and a season.

or by multiple herbivores, a subsequent herbivore may arrive and experience this induced phenotype during its stay on the plant, thereby prolonging the effects of plant phenotypic changes for the next couple of days or weeks (Figure 1). The timing of this subsequent arrival may have important consequences, as herbivore performance can be worse with a longer time elapsed since previous induction (Chapter5; Underwood 2012; Wang *et al.* 2015; but see Underwood 1998). However, when a second herbivore arrives after a much longer time after initial herbivory, the plant response could have already decayed, missing its effect on the second herbivore (see below; Underwood 1998; Karban 2011). In the *B. oleracea* system studied in this thesis however, the plant was rarely left alone by insects for more than a couple of hours or days during summer. A few days or weeks later after the first early-season herbivores, other arthropod community members can arrive on the plant, and in my thesis I identified that the timing of their arrival (early or later in the season) can change the subsequent development of that community (Chapter 7). The way in which timing of subsequent arrival of arthropod community members can affect the performance of subsequent community members depends on how a plant responds to multiple herbivory (Figure 2, see below) and how it prioritizes its responses to different attackers (Figure 3, see below).

Third, plant responses are likely maintained while the plant is under continuous herbivore attack during the season from early spring to late summer (Figure 1; van Zandt & Agrawal 2004b; Poelman *et al.* 2010), either at a certain level, or induced plant responses may continuously change direction and/or intensity (Figure 3, see below). However, after herbivory has ceased, such as during a winter period or between generations or abundance peaks of insect species, a lag in relaxation of plant response may occur (Underwood 1998; Karban 2011; Figure 1). The decay time may be much longer than the response lag (Karban 2011), perhaps due to a difference in predictability between a first and last herbivore of the season, for future herbivore attack. This predictability is high after the initiation of herbivory; the current herbivore will probably continue feeding for a while and newly arriving herbivores are likely expected after a first herbivory event at the beginning of the season (Karban & Adler 1996). The predictability whether new herbivores will arrive or not is much lower after herbivory has altogether stopped for a while; it might have been the last herbivore, or still a new herbivore may arrive (Karban & Baldwin 1997; Karban 2011). The ontogeny of a plant and/or seasonality (day length, temperature) may tune this predictability when it indicates to a plant that the end of a growth season, and thus the end of insect attack, is nearing (Boege & Marquis 2005; Cleland *et al.* 2007). Also the costs of herbivore attack will differ between a first and a last attacker of the season; damage to a young seedling may be more costly from a plant perspective than damage to older leaf tissue later in the season (Boege & Marquis 2005; Barton & Koricheva 2010).

Finally, plant responses may not completely decrease to before-herbivory levels after a period of herbivory absence (e.g. during winter; Figure 1). I have shown that

the resulting plant phenotype at the start of the new season may be different than the phenotype at the start of the season before, which may affect the colonisation of newly arriving insects from that moment onwards (Chapter 6). Next to such early-season legacy effects, also the community as a whole may cause a difference in the course of a plant phenotype during a summer period of insect attacks compared to the course of plant phenotype during the summer of the year before. This may be due to a cascade of plant-trait-mediated interactions between insects, that builds up on top of the plant-induced phenotype from the last year (van Zandt & Agrawal 2004b; Utsumi *et al.* 2010). This resulted in a legacy of previous insect interactions later in the season, which may affect future plant-mediated insect interactions on the same plant (Figure 1; Chapter 6).

In summary, plant responses that occur within minutes to days may influence insect-plant interactions that occur much later in the season or following years (Stam *et al.* 2014), *via* intermediate processes in plant physiology-insect interactions.

Consequences of timing between herbivory of insects from different feeding guilds for future herbivory

One of the aspects of timing in insect-plant interactions as discussed above, is that the order in which herbivores from different feeding guilds arrive and start feeding from a plant affect the plant phenotype, and the performance of subsequent insects (Chapters 3 and 4; Erb *et al.* 2011; Wang *et al.* 2014). Here I will discuss a theoretical mechanism of this. A possible explanation for the effects of order of herbivore arrival could be that induction of plant hormonal pathways at different moments causes different cross-talk patterns. Two of the main phytohormones involved in signalling responses to herbivory, jasmonic acid (JA) and salicylic acid (SA), have been shown to interact antagonistically when induced together (Thaler *et al.* 2002b; Diezel *et al.* 2009; Pieterse *et al.* 2012; Stam *et al.* 2014). However, the peak concentration of JA is often reached earlier than that of SA (de Vos *et al.* 2005; Ehlting *et al.* 2008; Mai *et al.* 2014; Figure 2a). Therefore, in theory, induction of the JA- and SA-pathway together by two different herbivores (for example a caterpillar and an aphid, respectively), may lead to a different timing of phytohormonal peaks when the induction occurs simultaneously or in sequence (Figure 2b-d; see also Koornneef *et al.* (2008) who experimentally tested part of the sketched situations). With simultaneous induction of the two pathways, the JA peak may occur before the SA peak, so that the interference of the JA pathway to the SA pathway may occur earlier than the reciprocal interference of the SA pathway to the JA pathway (Figure 2b). When the JA-inducing herbivore arrives first, this effect may be even more pronounced (Figure 2c). When the SA-inducing herbivore arrives first, however, the two phytohormonal peaks may occur simultaneously, or even in reversed order (depending on the time between herbivores, see Chapter 3; Figure 2d). This could lead to, for example, a more pronounced antagonism of the SA pathway on the

JA pathway. However, when the two moments of induction occur too far apart, interference may be absent if the window of interference is only short (Koornneef *et al.* 2008). The order of herbivore arrival may thus specifically influence a plant phenotype through differential cross-talk patterns. This may generate antagonistic interactions between herbivores feeding on the same plant with a negative effect for at least one of them (Erb *et al.* 2011; Mouttet *et al.* 2013), but in other cases, to synergistic responses in which at least one of the herbivores is positively affected (Kaplan & Denno 2007; Zhang *et al.* 2009; Soler *et al.* 2012a; Figure 1).

However, these two phytohormones are not the only ones involved in plant responses to herbivory (Ehlting *et al.* 2008; Robert-Seilaniantz *et al.* 2011; Erb *et al.* 2012), and the timing of phytohormonal regulation and cross-interaction may be more complex than sketched above (Mai *et al.* 2014). Furthermore, the sequence of events within the plant that lead to phytohormonal changes for a 'meaningful phenotype' that affects subsequently arriving insects, likely does not occur with a similar time course as the timing of induction by herbivores, which makes the situation even more complex (Erb *et al.* 2012; Barah & Bones 2014; Heidel-Fischer *et al.* 2014). Nonetheless, differential timing of herbivore-induced plant response signalling, including the production of phytohormones, may be the basis of the variation in performance of subsequent community members that has often been observed after sequential herbivore attack (Zhang *et al.* 2009; Erb *et al.* 2011; Soler *et al.* 2012a; Wang *et al.* 2014).

Prioritisation of plant responses to multiple herbivores from different feeding guilds

In addition to specific effects of timing between two herbivores, plants are specific in their responses to sequential feeding by different herbivore feeding guilds or species (de Vos *et al.* 2005; Bidart-Bouzat & Kliebenstein 2011; Ali & Agrawal 2014), and in this thesis I have seen that this has consequences for next arriving herbivores (Chapter 3) and other community members (Chapter 4). Moreover, we have identified that the history of herbivore attacks throughout the season (as well as plant ontogeny) is important in shaping the subsequent community composition (Chapter 7). Here I will discuss how, in theory, plants may respond to this continuous sequence of attacks by different herbivores (Figure 3) and thereby mediate effects of sequential dual herbivory to later community members.

A plant may change its response direction when one type of herbivore induces for example JA-based responses leading to the production of one set of secondary metabolites, structural change or other parts of the phenotypic 'syndrome', while another type of herbivore subsequently induces for example SA-based responses leading to another phenotypic 'syndrome' (Agrawal & Fishbein 2006). However, a plant may have several ways to prioritise its responses to a sequence of multiple herbivory (Stam *et al.* 2014). First, the plant response may be induced to a certain phenotypic 'syndrome' by the first attacker, but afterwards not change its defence

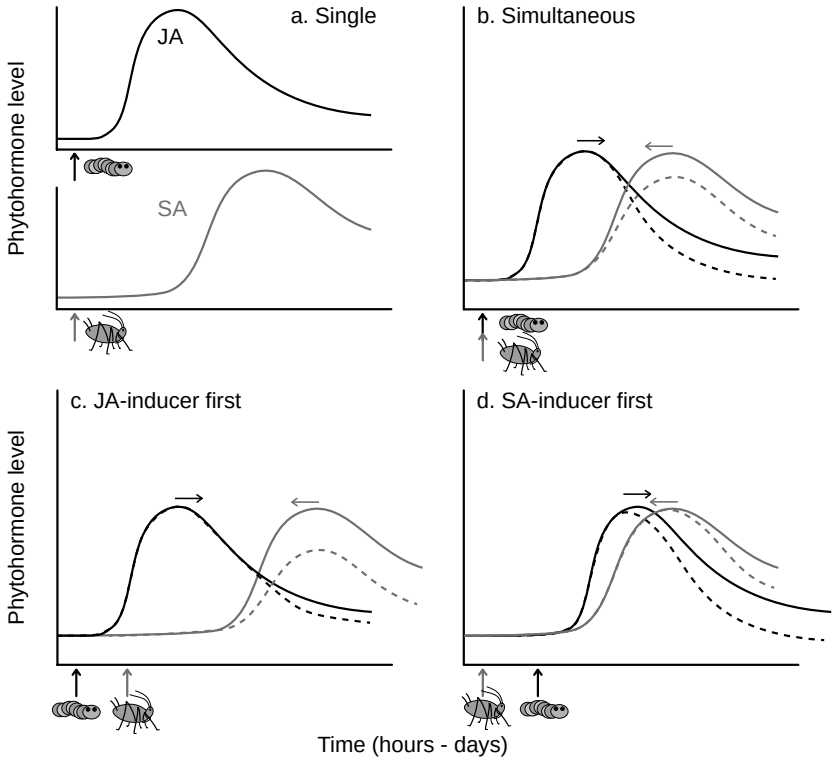

Figure 2. Timing of phytohormone induction and crosstalk in response to feeding by herbivores from different feeding guilds. The theoretical course of induction of two phytohormones, jasmonic acid (JA, black line) and salicylic acid (SA, grey line) by a JA-inducing and SA-inducing insect herbivore (vertical arrows) are shown in four different situations: a. each of the hormones induced separately; note that JA has an earlier peak than SA. b. JA- and SA-inducer arrive simultaneously, which causes interference or cross-talk (horizontal arrows) of the JA-pathway to SA-levels and *vice versa*, with the interference of JA to SA occurring first. c. JA-inducer arrives before SA-inducer, which causes interference of the JA-pathway to SA-levels to occur well before of the reciprocal interference. d. JA-inducer arrives after SA-inducer, which causes the interference of the JA-pathway to SA-levels to occur almost simultaneous as the reciprocal interference; or even later, depending on time between arrivals of the two inducers. Solid lines indicate hormone levels without interference between phytohormonal pathways, while dotted lines indicate phytohormone levels with antagonistic interference (cross-talk) of the JA-pathway to SA-production and *vice versa*.

phenotype anymore, regardless of sequential attackers (Figure 3a; Thaler *et al.* 2002b; Viswanathan *et al.* 2007; Wang *et al.* 2014). With such canalized response, the first herbivore early in the season is the dominant determinant of the plant phenotype throughout the rest of the season. This would be adaptive from the plant's perspective only when this first attacker is the most damaging, or subsequent

attackers are all negatively affected by this first induced plant defence response; or when costs of subsequent phenotypic change are too high. In contrast, a plant may continuously change direction of responses upon every new attack by a different type of herbivore. Every new herbivore would overrule the plant responses that were induced by the previous herbivore, which would make the phenotype highly variable and unpredictable over the season (Figure 3b; van Zandt & Agrawal 2004b; Erb *et al.* 2011; Coolen *et al.* 2016; Davila Olivas *et al.* 2016). This would be useful when each new attacker is very specific in which plant defence responses they experience as negative, and when the costs of plant phenotypic changes are low. A third scenario may be the intermediate situation: the plant initially responds to every new attacker, but eventually a phenotype is reached that integrates all previous responses (Figure 3c; Miller-Pierce & Preisser 2012). In this case, the plant phenotype would become less variable and less responsive to new herbivores as the season progresses (see also Boege & Marquis 2005), which would be adaptive to a very mixed and unpredictable attacking community, and when costs of plant phenotypic changes are high.

Each of the three scenarios assumes induced plant responses to last during a season of several weeks to months. Either an induced response that remains long-term (Figure 3a), or a chain of induced responses that each last only short but long enough to affect a subsequent herbivore which in its turn induces the next short-term response (Figure 3b) may explain season-long-lasting plant responses (Chapter 6; Utsumi *et al.* 2010; Wurst & Ohgushi 2015). However, both mechanisms pose limitations on the plant's physiology. A long-lasting induced response requires the maintenance of a response for several weeks or months, which may be costly (Koricheva *et al.* 2004; Vos *et al.* 2013a; Schuman & Baldwin 2016). On the other hand, a sequence of short-term changes requires a continuous, high plasticity of plant responses to allow phenotypic changes in a short time frame (high phenotypic plasticity; Ohgushi 2005; Utsumi *et al.* 2010), which may be physically or physiologically difficult, or it may be costly to produce many types of defences (Schuman & Baldwin 2016).

The intermediate scenario of integrative plant responses to different herbivores would perhaps best optimize these plant physiological limitations (McCall & Fordyce 2010; Schuman & Baldwin 2016). Also, this would fit best in theory on decreasing responsiveness to herbivory with increasing plant age, both due to changing available resources to defend, and changing costs of herbivory (Boege & Marquis 2005; although see Barton & Koricheva 2010). However, plants do seem to be able to respond to single herbivores later in the season within the background of an established insect community (Chapter 7), which would more point towards a (partial) overruling scenario, because in the integration scenario, plant responses become less variable and the phenotype may eventually not change at all anymore.

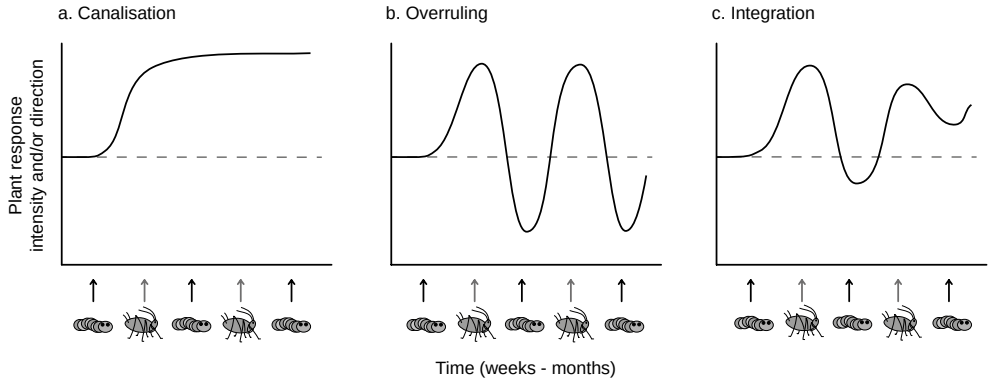

Figure 3. Different ways of prioritisation of plant responses to a sequence of multiple herbivores. Three possible scenario's describing the intensity and/or direction of induced plant responses to herbivory by different herbivore species. The arrival of herbivores from different types (species, feeding guilds) are depicted by black or grey vertical arrows. a. Canalisation: the first arriving herbivore induces a response, which thereafter does not change, regardless of subsequent herbivory. b. Overruling: each arriving herbivore induces a plant response in a different direction, overruling the response induced by the previous herbivore. c. Integration: plant responses change direction after every new herbivore, but slowly converge towards one specific direction, depending on all previous herbivore attacks combined.

Adapting in a dynamic multi-herbivore - plant community, for both plant and insects

An insect-plant community is highly dynamic over time (Stam *et al.* 2014), and even varies between plant populations (Chapter 4; Li *et al.* 2016). To be able to survive and reproduce in such a dynamic environment, both the plant and the insects should have adapted to deal with continuous changes.

From the plant perspective, they are continuously under attack by herbivores with very different characteristics: their feeding guild, host specialisation, seasonality, size and densities all may differ (e.g. Denno *et al.* 1995; Agrawal 2000; Bidart-Bouzat & Kliebenstein 2011; Gols *et al.* 2011; Kroes *et al.* 2015). Next to herbivores, also insects from higher trophic levels (predators and parasitoids) occur, as well as mutualists such as pollinators (Schoonhoven *et al.* 2005; Kessler & Halitschke 2009; Kessler *et al.* 2010; Lucas-Barbosa *et al.* 2013; Stam *et al.* 2014). The wild cabbage plants in this study experienced interactions with well over 30 different insect species (see supplementary Table 1 of Chapter 4 and Table 1 of Chapter 7) from May to early October, and similar again in the following year. In theory, each of the herbivorous insects could induce a different plant response which all other insects may face, although plants may prioritize their responses to each insect in different ways (Figure 3).

Plant responses are functional for the plant if they eventually lead to a higher

plant reproduction (defensive responses; Poelman 2015; Schuman & Baldwin 2016). However, a potentially costly resistance response to an herbivore from a highly dynamic community would only be beneficial if it indeed negatively affects the most damaging future attackers, and does not create a net positive effect to other herbivores (Kessler & Baldwin 2004; Voelckel & Baldwin 2004; Zhang *et al.* 2009; Soler *et al.* 2012a). In a dynamic insect community, the predictability of who is the next arriving insect might be low (Poelman & Kessler 2016). Therefore, a plant resistance response to an herbivore species that afterward does not arrive anymore, might miss its target against those future herbivores, or would simply be a waste of resources (Schuman & Baldwin 2016). In contrast, a resistance response to a cue that is highly predictable for future damage is favourable for a plant, for instance a resistance response to eggs of a voracious caterpillar (Pashalidou *et al.* 2015). As a parallel example, learning behaviour in a parasitic wasp to find its host was only fast when the learned cues were highly predictable for finding a next host; otherwise the connection between cue and reward was soon forgotten (Hoedjes *et al.* 2011). Therefore, plant resistance responses to individual insects in a community might only be 'useful' if the current damage predicts future damage, either by the same herbivore or by a future herbivore. However, how predictable insect communities are, either due to fixed sequences of insect arrival every year or due to predictable insect colonisations based on induced plant phenotypes, is a field of research that is only just starting (Poelman & Kessler 2016; Schuman & Baldwin 2016).

From the perspective of insect community members, the changes in plant phenotype in response to herbivory, as well as to abiotic factors, pose a dynamic environment to plant-associated insects. The insects that have tight interactions with a plant should be adapted to these continuous environmental changes in order to survive and reproduce. Much of the research on host specialisation of insects has addressed this question; specialists are predicted to have adapted to a few plant species with their specific range of phenotypic variation, while generalists have adapted to a whole range of different host plant species, which each have very different traits (Schoonhoven *et al.* 2005; Bidart-Bouzat & Kliebenstein 2011; Ali & Agrawal 2012).

Plants and insects thus continuously respond to each other to adapt to changes from the environment (either insect attack or plant phenotype) and retain a high fitness. The feedback loop of plant responses to insects, and insect responses back to induced-plant phenotype, could eventually lead to eco-evolutionary dynamics (Ohgushi 2016). Herbivory induces plant phenotypic plasticity, which feeds back to the insect community via plant-mediated interactions between insects (Chapter 4; Utsumi *et al.* 2010; Ohgushi 2016). The insect community in its turn can feed back to plant phenotypic plasticity, either directly via functional traits of the community, or indirectly via plant-mediated indirect insect interactions (Utsumi *et al.* 2013;

Ohgushi 2016; see also Whitham *et al.* 2006). Eventually, herbivores shape part of their environment by inducing plant phenotypic changes, and plants influence their associated insect community by responding to the insects that occur on the plant (Whitham *et al.* 2006; Ohgushi 2016).

Conclusions and perspectives for future research

Two or more herbivore species feeding on the same plant induce different plant responses than the sum of responses to each of the herbivores alone (Stam *et al.* 2014). In this thesis I have shown that this has consequences beyond the plant itself, and also affects other insects that arrive on this plant with a dual-herbivore induced phenotype. The choice and performance of a subsequent feeder was affected, as well as the colonisation dynamics of a whole insect community. Finally, this had a feedback effect to the insect community a year later, and to the fitness of the plant itself. Both changes in induced plant traits (phenotypic plasticity) as well as changes in plant age on top of the changes induced by the community until that moment, play a role in conferring early-season induction to later-season community dynamics. In the following section I highlight some research questions on multiple insect - plant interactions that still remain to be tested and that would further advance our understanding of plant-insect community organisation in a more natural, multi-herbivore context.

Both the order of arrival and the time interval between two arriving herbivores is important in shaping plant response, and thus the phenotype that later insects face. The mechanism of these patterns likely have to be sought in the kinetics of plant physiology: when is which response induced, how do the responses induced by different herbivores interfere, and how does this pattern change with varying time lags between herbivore arrivals (e.g. Figure 2 and nuances beyond)? Also more detailed studies of the plant response lag after the onset of herbivory, and especially, the decay lag when herbivory has ceased, would further allow to connect short-term plant response processes with insect community dynamics that occur over weeks to months, sometimes even years.

A plant generally interacts with a large variety of insects, throughout the season. The first one or two insect species that feed on the plant early in the season can have a considerable impact on the development of the insect-plant community during the rest of the season (Chapter 4; Viswanathan *et al.* 2007; Poelman *et al.* 2008a), but plants do still seem to respond to individual insects mid-season, when they have already experienced a history of the insect community (Chapter 7). Future studies should focus on how plants prioritise their responses to multiple insects, not only early in the season, but also later on. To unravel prioritisation patterns, we need to

know which plant physiological limitations and costs are the most important, and second, whether the predictability of insect community compositions over time play a role in determining the plant's response strategy (Poelman & Kessler 2016).

Furthermore, the question arises whether insect community dynamics really are predictable, and if so, what underlies this? Is the sequence in which insect species arrive on a plant fixed, driven by their phenology, or can insects make predictable choices of colonisation based on the plant-mediated cues of which insects are or were already present on the plant? To go from two herbivores to truly multiple herbivore – plant interactions systems (Poelman 2015), we need both detailed ecological, as well as mathematical modelling studies to get the first grip on unravelling all the individual interactions within a large, dynamic and complex interaction web.

Finally, plant populations were shown to differ in their responses to multiple herbivory, and we know that plant populations or species can vary in how large their induced responses are to single herbivores (Gols *et al.* 2008b; Agrawal *et al.* 2014). Is this variation in the extent to which plants are able to respond to herbivory, their inducibility, related to how predictable their associated community of inducers is? In a highly dynamic and unpredictable community, a high inducibility (and low levels of constitutive defence) may be useful in order to respond to unexpected damagers (but save resources if they do not (yet) show up). In contrast, a less dynamic, more predictable insect community would perhaps favour a lower inducibility (but higher levels of constitutive defence), to be prepared for damaging insects that are likely to arrive (Karban & Baldwin 1997; Poelman & Kessler 2016). Answering these questions will require studies with multiple plant populations or plant species with a range of different associated insect communities that differ in their properties, such as the predictability of insect arrival order and the fitness constrains that damage of individual community members pose to the plant.

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Summary

Plants interact with many organisms around them, and one of the most important groups that a plant has to deal with, are the herbivores. Insects represent the most diverse group of herbivores and have many different ways of using the plant as a food source. Plants can, however, defend themselves against those herbivores, either by constitutive defences, or by traits that are induced upon herbivory. These traits, such as the formation of more trichomes or the production of secondary metabolites, can deter an insect herbivore in its decision to eat from the plant, can be toxic, or otherwise hamper the insect to feed, grow or reproduce. The way a plant responds to herbivory is very specific, depending on the feeding mode or the species of the attacking insect. Furthermore, plant responses to dual herbivory differ from the sum of responses to each herbivore alone. Also the time and order at which multiple insects arrive on a plant, influence the plant's response. Finally, plant species or populations can show different responses to herbivory. Altogether these factors result in a plant phenotype that the attacking herbivore has to deal with. In addition to the attacker, also subsequently arriving insects will be affected by a change in plant phenotype. Because plants and insects can respond to each other in a continuous chain of interactions, an herbivore early in the season can indirectly affect the later-season community composition through the induced plant response. However, we know only little about the consequences of a dual-herbivore induced plant phenotype for subsequent feeders, and ultimately, the effects on the assembly and dynamics of an insect community as a whole.

The aim of this thesis project was to study the consequences of feeding by multiple insects from the same plant, not only to a subsequent herbivore, but also to the dynamics of a whole insect community over the course of a growing season, and beyond. Furthermore, I studied how the order of herbivore arrival and timing of arrival affected both a next herbivore's choice and performance in a greenhouse setting, as well as the development of the whole insect community in the field. In addition, I studied how plant populations vary in induced responses, have specific plant-mediated interactions among insect herbivores, and how long these induced responses influence the insect community and plant fitness. Finally, I identified non-additive effects of the history of insect attacks and plant ontogeny to the future insect community.

In the first chapter of this thesis, I introduce the study system. For this project I used several populations of the wild cabbage plant, *Brassica oleracea*. This is an herbaceous perennial plant that flowers from the second growing season onwards, and supports a large and diverse above-ground arthropod community of more than thirty different species. The plant belongs to the family of Brassicaceae, which is known for the biosynthesis of a group of secondary metabolites, the glucosinolates. These metabolites may deter insects, although some insect species use it as a feeding cue. Two specialist insect herbivores from different feeding guilds, the

caterpillar of the diamondback moth *Plutella xylostella* and the cabbage aphid *Brevicoryne brassicae*, were used to study their effect as early-season inducer of plant responses either alone or in combination. The caterpillar of the generalist cabbage moth, *Mamestra brassicae*, was used in bioassays to assess the effects of the induced plant phenotype by single or dual herbivory. Furthermore, in three chapters (Chapters 4, 6 and 7) I have closely studied the composition of the naturally occurring insect community throughout the season for one or two years in a common-garden field setting. In the last of these three chapters, I used the caterpillars of the specialist cabbage white, *Pieris rapae*, to induce plants at different moments of their ontogeny, while excluding the insect community for varying periods of time by a net or exposing plants to their natural insect community.

In an elaborate literature review, I and my collaborators concluded in chapter 2 that plant responses to dual herbivory evoke different plant responses than the sum of each herbivore alone. This has consequences at all levels from arthropod community assembly to the choice and performance of individual insects. The mechanisms of plant responses to dual herbivory are found in gene expression, hormone production and other molecular processes within the plant. All these aspects of interactions between insects and plants occur and are connected at different time scales.

To follow up on the question how timing plays a role in dual herbivory, we varied the time between, as well as the order of arrival of aphids and/or caterpillars on a plant. We observed that both affected the preference and performance of a subsequently feeding caterpillar (Chapter 3). *Mamestra brassicae* performed better on plants with a longer time interval between the first and second feeder. Also in a field setting (Chapter 4), the order of herbivore arrival early in the season affected the insect community composition later in the season in two different years, likely through a chain of indirect insect interactions. In this field study, the plant population influenced the outcome of early-season herbivory to later community dynamics. In chapter 5 we found that three plant populations in response to simultaneous aphid and caterpillar attack differed in the expression of two genes that are important for the regulation of herbivore-induced responses. Also, the production of one of two important plant hormones, salicylic acid, responded differently to single or dual herbivory in a unique pattern for each of the plant populations. These different plant responses subsequently negatively affected a next caterpillar on the same plant; *M. brassicae* growth was impaired on plants which had been fed upon by both aphids and caterpillars, in comparison to control plants. These field and greenhouse studies thus show the implications of dual herbivory beyond effects in the plant; it affects subsequent herbivory, and through a chain of plant-mediated insect interactions, the dynamics of a whole insect community.

In the sixth chapter we show that variation in insect community dynamics can last beyond the moment that the insects were present, even across years. In this field study, the naturally occurring carnivore community influenced the carnivore community composition a year later. Importantly, the herbivore community affected plant fitness across years (but not within years). We propose that such legacy effects are mediated by plant traits, which vary upon insect induction in the first year, and affect the insect community in the next year.

Finally, the history of all insect attacks to a plant up until that moment shape the future insect community by influencing the colonisation of insect species on the plant. Moreover, also plant ontogeny plays a role in shaping the insect community; plant-mediated responses to herbivory at different plant ages resulted in different insect colonisation rates. The most important conclusion from this last data chapter (Chapter 7) is that the two processes, insect community history and plant ontogeny, are non-additive and affect the colonisation of insect species in the same (synergistic) or opposite (antagonistic) direction.

By framing my study results in a time line from minutes to months to years, I show in the general discussion (Chapter 8) that the consequences of dual herbivory for subsequently arriving insects are connected at different time scales. Plant responses to herbivory can occur within hours to days, which affect herbivore choices and performance in the following days and weeks. In their turn, variation of a few days in arrival time of insects may change how plants respond and prioritize their responses to insects throughout the rest of the season. The insects that subsequently arrive on a dual-herbivore induced plant may change the plant phenotype even further and through a chain of insect-plant interactions, the effects on the insects and the plant can last throughout the season, and even across seasons. Furthermore, various factors such as the species of the attacking insect and its feeding guild, the timing after previous attack and the plant age at which herbivory occurs, as well as the genotypic background of the plant, all affect the outcomes of dynamic insect-plant interactions.

The results presented in this thesis thus contribute to the knowledge and interpretation of plant interactions with multiple herbivores. As plants are seldom attacked by a single herbivore, this implies that we have to take into account that multiple herbivory is not the same as the additive effects of single herbivores, and that this has long-lasting consequences for the insect community and the plant. To further understand how plants and insects have adapted to such a dynamic environment, I suggest future research to focus even more on the kinetics of plant physiological responses to dual attack, and to aim at answering the question of how predictable insect communities on a plant really are.

Acknowledgements

Curriculum vitae

Publications

Education statement

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Liefs,
Jeltje

Curriculum vitae

Jeltje Marlijn Stam was born on the 3rd of April 1987 in Groningen, the Netherlands, where she followed her primary and high school education. In 2006 she started her BSc in Biology at the Wageningen University. Already from the beginning onwards an interest in entomology was evoked after following a series of evening lectures on 'Insects and Society', and later a BSc thesis in entomology followed. During her MSc studies she continued specialisation in this direction by conducting a thesis on the winter survival of the harlequin ladybird, *Harmonia axyridis*, at the laboratory of Entomology of the Wageningen University. As a sidestep, she then did an MSc minor thesis at the department of Terrestrial Ecology of the Netherlands Institute of Ecology (NIOO-KNAW) where she investigated the feedback of soil fauna to exotic plants that have varying residence times in The Netherlands. She concluded the MSc programme with an internship at the Institut de Recherche en Biologie Végétale (IRBV) of the Université de Montréal, Canada, which was, back to entomology, on the demographics of a parasitoid of the soybean aphid, a pest insect. Throughout both her BSc and MSc studies, she was involved in teaching practical courses such as ´Growth, development and reproduction of plants´, and ´Behaviour and Environment´.

After obtaining her MSc degree with distinction (*cum laude*), she started her PhD project in January 2012 at the Laboratory of Entomology of Wageningen University, supervised by Dr Erik H. Poelman and Prof. Dr Marcel Dicke. During this project in which she collaborated with two other PhD candidates, she studied the ecological aspects and consequences of multiple insect herbivory on a perennial plant. The results are described in the book resting in your hands. In addition to conducting experiments, she was involved in organising the Wageningen Evolution and Ecology Seminars, and she organised a week-long PhD study trip to Switzerland. Also during her PhD programme, she was involved in teaching, for example the course ´Ecology I´, and supervised various BSc and MSc students during their thesis projects.

In April 2016 she started a post-doc research project at the Swedish University of Agricultural Sciences (SLU) in Alnarp, Sweden, where she works in the 'strawberry lab' of Dr Johan A. Stenberg on herbivore - pollinator interactions on woodland strawberry.

Publications

Stam, J.M., Chrétien, L., Dicke, M. & Poelman, E.H. Temporal dynamics of plant response to attack by two herbivores on *Brassica oleracea* affects preference and performance of a third herbivore, *Mamestra brassicae*. *Submitted*

Chapter 3 of this thesis.

Stam, J.M., Dicke, M. & Poelman, E.H. Order of herbivore arrival on wild cabbage populations influences subsequent arthropod community development. *Submitted*

Chapter 4 of this thesis.

Kroes, A.*, Stam, J.M.*, David, A., Boland, W., van Loon, J.J.A., Dicke, M. & Poelman, E.H. (**Accepted**) Plant-mediated interactions between two herbivores differentially affect a subsequently arriving third herbivore in populations of wild cabbage. *Plant Biology*, DOI: 10.1111/plb.12490.

Chapter 5 of this thesis.

Li, Y., Stam, J.M., Poelman, E.H., Dicke, M. & Gols, R. (**2016**) Community structure and abundance of insects in response to early-season aphid infestation in wild cabbage populations. *Ecological Entomology* 41: 378-388.

Speek, T.A.A., Schaminée, J.H.J., Stam, J.M., Lotz, L.A.P., Ozinga, W.A. & van der Putten, W.H. (**2015**) Local dominance of exotic plants declines with residence time: a role for plant–soil feedback? *AoB Plants* 7.

Stam, J.M.*, Kroes, A*., Li, Y.*, Gols, R., van Loon, J.J., Poelman, E.H. & Dicke, M. (**2014**) Plant interactions with multiple insect herbivores: from community to genes. *Annual Review of Plant Biology* 65: 689-713.

Chapter 2 of this thesis.

Raak-van den Berg, L.C., Stam, J.M., de Jong, P.W., Hemerik, L. & van Lenteren, J.C. (**2012**) Winter survival of *Harmonia axyridis* in The Netherlands. *Biological Control* 60: 68-76.

*These authors contributed equally to the publication

Education Statement of the Graduate School Experimental Plant Sciences

Issued to: **Jeltje M. Stam**
Date: **7 October 2016**
Group: **Laboratory of Entomology**
University: **Wageningen University & Research**

1) Start-up phase	*date*
► **First presentation of your project**	
PhD presentation; 'Multiple herbivore plant attack consequences for the insect community'	Nov 06, 2012
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Review: "Plant Interactions with Multiple Insect Herbivores: From Community to Genes", *Annual Review of Plant Biology* 65: 689-713, 2014 (Doi: 10.1146/annurev-arplant-050213-035937)	Nov-Aug 2013
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student day, University of Amsterdam	Nov 30, 2012
EPS PhD student day, Leiden University	Nov 29, 2013
► **EPS theme symposia**	
Theme 2 'Interactions between Plants and Biotic Agents' (combined with Willie Commelin Scholten day), Wageningen University	Feb 10, 2012
Theme 2 'Interactions between Plants and Biotic Agents' (combined with Willie Commelin Scholten day), Utrecht University	Jan 24, 2013
Theme 2 'Interactions between Plants and Biotic Agents' (combined with Willie Commelin Scholten day), Utrecht University	Feb 20, 2015
Theme 2 'Interactions between Plants and Biotic Agents' (combined with Willie Commelin Scholten day), Leiden University	Jan 22, 2016
► **Lunteren days and other National Platforms**	
NAEM (NL Annual Ecology Meeting) 2012, Lunteren, NL	Feb 07-08, 2012
NAEM (NL Annual Ecology Meeting) 2013, Lunteren, NL	Feb 05-06, 2013
NAEM (NL Annual Ecology Meeting) 2014, Lunteren, NL	Feb 11-12, 2014
NAEM (NL Annual Ecology Meeting) 2015, Lunteren, NL	Feb 10-11, 2015
NAEM (NL Annual Ecology Meeting) 2016, Lunteren, NL	Feb 10, 2016

Entomology day NEV, Reehorst Ede 2012	Dec 14, 2012
Entomology day NEV, Reehorst Ede 2013	Dec 13, 2013
Entomology day NEV, Reehorst Ede 2014	Dec 19, 2014
► **Seminars (series), workshops and symposia**	
YELREM 2012 (Yearly Entomology Laboratory Research Exchange Meeting), Wageningen UR	May 30, 2012
7th Workshop Plant-Insect Interactions, Leiden, NL	Nov 28, 2012
Seminar by Dr. Nancy Schellhorn (Spatial Ecology, CSIRO; Brisbane, Australia); Wageningen UR	Oct 03, 2012
Seminar on Gender Balance at Wageningen UR, Wageningen	Nov 13, 2012
WEES-lecture by Dr. Ron Ydenberg (Simon Fraser University, Canada), Wageningen UR	Nov 22, 2012
WEES-lecture by Dr. Nicole van Dam (Radboud University Nijmegen); including preceeeding Master Class by Dr. Nicole van Dam; Wageningen	Dec 20, 2012
EPS Expectations Day 2013; “Creativity and inspiration in science", Wageningen	Feb 01, 2013
YELREM 2013 (Yearly Entomology Laboratory Research Exchange Meeting), Wageningen UR	May 17, 2013
Wageningen Young Academy Lecture Prof. Frans de Waal, “The Bonobo and the Atheist” (Wageningen UR)	Jun 26, 2013
EPS Symposium 'From Model System to Ecology and Evolution’, Leiden, NL	Aug 29, 2013
8th Workshop Plant-Insect Interactions, Wageningen UR	Sep 24, 2013
Plant Sciences Seminar: “Metabolomics in the lab: a myriad of applications”, Robert Hall and Nicole van Dam	Oct 08, 2013
Mini-symposium “How to Write a World-class Paper”, organised by Wageningen UR Library and Elsevier	Oct 17, 2013
Seminar Peter Biedermann (IEE, University of Basel), “Cooperation under the bark” – fungi rearing Ambriose beetles; Wageningen UR	Nov 26, 2013
Seminar Dr. Niklas Wahlberg (Laboratory of Genetics, Department of Biology, University of Turku, Finland); ‘The 215 million years of Lepidoptera diversification: lessons from an ever changing world’, Wageningen UR	Dec 12, 2013
WEES-lecture Martine Maan (NWO Veni Feloow, Groningen University); “Speciation in colour: diversifying natural and sexual selection in cichlid fish and poison frogs”; Wageningen UR	Dec 19, 2013
WPC (Wageningen PhD council) lecture, "The secret of a successful PhD: Making the implicit explicit"	Feb 06, 2014

YELREM 2014 (Yearly Entomology Laboratory Research Exchange Meeting), Wageningen UR	May 21, 2014
Seminar Pieter Zuidema; "An editor's perspective of the reviewing process"; Wageningen	May 27, 2014
Wageningen Young Academy Public lecture & debate by Hans Clevers: "The future of Science" (Wageningen UR)	Jun 12, 2014
WEES-lecture by Joy Bergelson (Chicago University, USA) "Maintaining an ancient balanced polymorphism for resistance amidst diffuse interactions", Wageningen UR	Sep 26, 2014
9th Workshop Plant-Insect Interactions, Utrecht, NL	Nov 03, 2014
PE&RC Day 2014 "Optimization of Science: Pressure and Pleasure", Wageningen UR	Nov 13, 2014
"Current themes in Ecology", organised by NERN, Wageningen UR	Nov 20, 2014
WEES lecture + preceeding Master Class by Koos Biesmeijer (Scientific Director at Naturalis Biodiversity Centre, Leiden); "On Bees, Pollination and Food Security"	Dec 18, 2014
WEES lecture by Prof. Kevin Foster (Evolutionary biology, Oxford, UK), "The evolution of cooperation and competition in microbes"	Jan 22, 2015
NIOO seminar by Prof. John Liu (Director 'Enviromental Education Media Project'), 'The art of healing the earth', NIOO-KNAW, Wageningen, NL	Jan 26, 2015
WEES lecture by Doug Landis (Professor of Entomology at Michigan State University); "Redesigning Agricultural Landscapes for Multiple Ecosystem Services	Feb 19, 2015
CSA seminar by Prof. Velemir Ninkovic (Uppsala University SLU), 'Plant-plant communication'	Mar 05, 2015
EPS Seminar by Prof. dr. Florian Schiestl, 'Evolution of floral signals in plants: mechanisms and consequences', WageningenUR	Mar 12, 2015
WEES lecture by Prof. Hanna Kokko (University of Zürich, Switzerland), "Males exist. Does it matter?"	Mar 19, 2015
Wageningen PhD Symposium, "Connecting Ideas, Combining Forces"	May 06, 2015
WEES lecture + preceeding Master Class by Yolanda Chen (Assistant Professor at the Uiversity of Vermont, USA); "Anthropogenic effects on insect-plant interactions in agriculture: crop domestication and global invasions"	May 21, 2015
YELREM 2015 (Yearly Entomology Laboratory Research Exchange Meeting), Wageningen UR	Jun 10,2015

WEES lecture by Julien Veraldi (Assistant Professor of the Laboratory of Evolution Biology and Biometric, Claude Bernard University Lyon 1, France), "Behavioural manipulation and horizontal gene transfer in a virus-parasitoid interaction"	Oct 22, 2015
Seminar by Prof. Laura Grenville-Briggs, Swedish University of Agricultural Sciences (SLU), Alnarp, Sweden; "Molecular Oomycete-Host Interactions: The Good, the Bad and the ugly"	Feb 19, 2016
► **Seminar plus**	
► **International symposia and congresses**	
15th SIP-meeting, Neuchâtel, Switzerland	Aug 17-22, 2014
Anual meeting of Britisch Ecological Society (BES), Edinburgh, UK	Dec 13-16, 2015
► **Presentations**	
8th Workshop Plant-Insect Interactions, Wageningen, NL (Talk); "Will plants never get tired of defending? – Time and sequence of herbivores affect plant defence and subsequent community"	Sep 24, 2013
15th SIP-meeting, Neuchâtel, Switzerland, (Talk); "Order of arrival of early season herbivores affect the subsequent insect community"	Aug 21, 2014
9th Workshop Plant-Insect Interactions, Utrecht (Talk); "Order of arrival of early season herbivores affect the subsequent insect community"	Nov 03, 2014
NAEM 2015, Lunteren, NL (Talk); "Plant-mediated interactions between multiple herbivores affect a subsequently arriving herbivore in populations of wild cabbage"	Feb 11, 2015
Anual meeting of British Ecological Society, Edinburgh, Scotland (Talk); "Now or later? Legacy effects over years of arthropod community and consequences for plant fitness"	Dec 15, 2015
NAEM 2016, Lunteren, NL (Talk); "Legacy effects of arthropod community to next year's community composition and plant fitness, mediated by perennial plants"	Feb 10, 2016
► **IAB interview**	
► **Excursions**	
PhD excursion Entomology Switzerland (Neuchâtel, Lausanne, Basel)	Oct 27-Nov 02, 2013
Subtotal Scientific Exposure	*19.0 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
Course 'Introduction to R'	Jun 10-11, 2013
Course 'Mixed Linear Models' (Statistics)	Jun 20-21, 2013

Course 'General Linear Models' (Statistics)	Jun 16-17, 2014
Course 'Multivariate Analysis' (Statistics)	Oct 14-16, 22-23, 2014
► **Journal club**	
PhD lunch meetings; Entomology, Wageningen University	2012-2015
Insect-Plant Interactions-lunch meetings; Entomology, Wageningen University	2012-2015
► **Individual research training**	
Subtotal In-Depth Studies	*6.3 credits**

4) Personal development	*date*
► **Skill training courses**	
Competence assessment	Aug 28 & Sep 28 2012
Information Literacy (including EndNote course)	Dec 04, 05, 19 2012
Course 'Techniques for Writing and Presenting a Scientific Paper (TWP)"	Dec 03-06, 2013
Course "Afstudeervak organiseren en begeleiden"	Dec 09-10, 2013
Course 'Guide to Scientific Artwork' (by WUR library)	Mar 23-24, 2015
Course 'Voice Matters - Voice and Presentation Skills Training (V&PT)'	Apr 07, 21, 2015
PhD workshop caroussel	Apr 17, 2015
Course 'Last Stretch of the PhD'	May 22, 2015
Course 'Adobe Indesign Essential Training'	Sep 29-30, 2015
► **Organisation of PhD students day, course or conference**	
Organising week-long PhD trip, 27 October - 2 November 2013, Switzerland	Sep 2012-Nov 2013
Secretary of WEES (Wageningen Ecology and Evolution Seminars)	Sep 2014-Sep 2015
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*7.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**40,4**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits

** A credit represents a normative study load of 28 hours of study.*

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