Caught between Friends and Foes

Plant-mediated interactions between herbivores and flower visitors

Quint Rusman

Propositions

1. Herbivore trait variation has important consequences for how herbivore and flower visitor networks are linked.
   (this thesis)

2. Timing of herbivory during plant ontogeny influences plant fitness.
   (this thesis)

3. Peer review is a lottery.

4. The appreciation of novel rather than thorough makes science superficial.

5. Non-significant results are undervalued.

6. Ecosystem services are not a valid argument to defend the conservation of rare species.

7. Political correctness is a paradox.

8. This thesis offends at least one person.

Propositions belonging to the thesis, entitled

Caught between friends and foes – plant-mediated interactions between herbivores and flower visitors

Quint Rusman
Wageningen, 12 April 2019

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Plant-mediated interactions between herbivores and flower visitors

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Thesis

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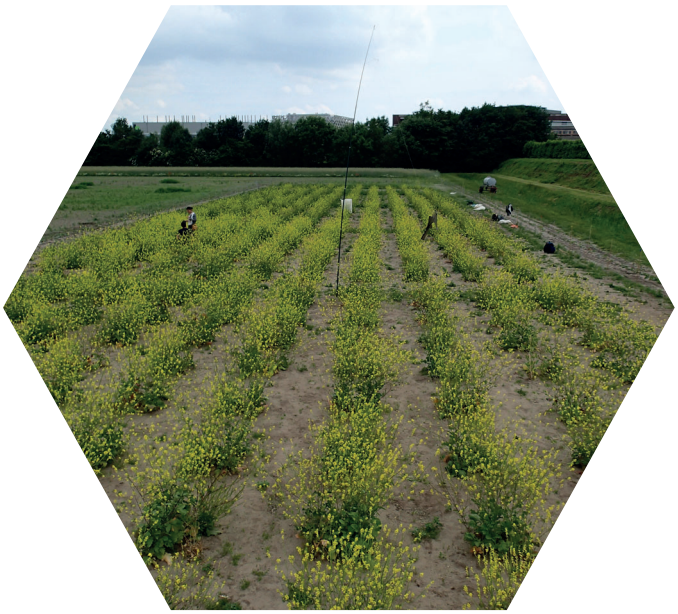

Chapter 1

General introduction

The fundamental aim of ecology is to understand how organisms interact with their environment. Some of the broader ecological questions today concern the dynamics of complex interactions among species in ecosystems and environmental change, and the interplay between ecology and evolution in complex multispecies communities (Sutherland et al. 2013). Plants form the foundations of many ecosystems. About 400,000 species of plants are known to science, and the majority of these species are flowering plants (Soltis and Soltis 2014). Half of the estimated 4-10 million species of insects feed on plants tissues such as leaves, roots, and flowers (Schoonhoven et al. 2005). That is at least 5 herbivorous insect species for each plant species! Most flowering plants, 85 percent, also provide food in the form of nectar and pollen for flower-visiting insects, which in turn aid in the reproduction of these plants (Ollerton et al. 2011). To maximize their contribution to the next generation, flowering plants need to deal with antagonists while also engaging in interactions with mutualists (Herrera et al. 2002, Grass et al. 2018). Because plants are under selection to maximize fitness in a community context including antagonistic and mutualistic insects, plant-insect systems provide excellent models to study ecological and evolutionary dynamics in complex multispecies communities.

Plant antagonists range from large mammals to tiny insects, and microscopic bacteria and viruses. Each of these attackers may differ in mode of attack as well as the fitness costs associated with the attack (Bernays 1998, Wise and Rausher 2013). For example, caterpillars can cause extensive damage by chewing tissues between powerful mandibles, consuming leaves and flowers partly or completely. In contrast, aphids feed stealthily on the phloem sap using slender stylets with minimal tissue damage. Some insects exclusively feed on roots or pollen grains, whereas others start feeding on leaves and move to the flowers during their development (Bernays 1998, Lucas-Barbosa et al. 2013). Because plant tissues have different fitness values to a plant (McKey 1974, Stamp 2003), antagonist can impact plant fitness differently depending on their feeding behaviour (McCall and Irwin 2006, Wise and Rausher 2013). To diminish adverse effects of antagonist attack, plants evolved various defence strategies (Schoonhoven et al. 2005). These include direct defences, such as morphological structures and toxic chemicals that harm or repel the herbivore directly, and indirect defences, such as volatile compounds that attract the natural enemies of herbivores. Defences can be constitutively present, and/or inducible upon herbivore attack. Inducible defences allow responses to be fine-tuned to the specific attacker, save metabolic costs of resistance in the absence of herbivores, reduce the possibilities for herbivores to adapt to a fixed plant phenotype, and facilitate information exchange between the plant and its environment (Karban and Baldwin 1997, Karban 2011, Kessler 2015). Plants can recognize specific attackers by their feeding mode, salivary constituents, and patterns of damage (Erb et al. 2012). It is well known that leaf-chewing and sap-feeding herbivores induce different phytohormonal signal-

transduction pathways in leaves that cascade into different plant phenotypic responses (Ali and Agrawal 2012, Thaler et al. 2012). In general, leaf-chewing herbivores induce the jasmonic-acid (JA) pathway, whereas sap-feeding herbivores induce the salicylic-acid (SA) pathway. The plant phenotypic response is further influenced by a range of other phytohormone-mediated pathways (Erb et al. 2012). Inducible plant responses are usually expressed in both local and systemic tissues (Hilleary and Gilroy 2018). As a result, plant responses to antagonists may alter plant interactions with other community members, such as mutualistic organisms.

Plant mutualists belong to different trophic levels, from mycorrhizal fungi and pollinators that benefit the plant directly (Morris et al. 2007), to predators, parasitoids, entomopathogens, and soil microbes which benefit plants by attacking or competing with plant antagonists (Bronstein and Barbosa 2002). Plants evolved various organs and signals to attract mutualistic organisms. In the angiosperms, flowers are among the most conspicuous of such organs. Flowers provide food in the form of pollen and nectar, and advertise these treats through odours and bright colours, which attract pollinators. In return, pollinators fertilize the plant by transporting pollen grains, which contain the male gamete, from one flower onto the stigma of another, thereby completing an essential step in the reproduction of the plant. Direct damage to flowers and plant phenotypic changes in response to herbivore attack can alter the expression of flower traits, and influence the behaviour of flower visitors (Lucas-Barbosa 2016, Jacobsen and Raguso 2018).

Plant strategies to maximize one interaction may come with costs by altering other interactions. Indeed, conflicts in interactions between plants and their antagonists and mutualists may occur. To reduce the consumption of flowers by herbivores, resource allocation of plants to defensive compounds in flowers may go at the expense of energy for additional flower or fruit formation (Herms and Mattson 1992, Quesada et al. 1995, Poveda et al. 2005), and pollinators may be deterred by flower defensive traits (Adler 2000, Kessler and Halitschke 2009, Schiestl et al. 2014). Moreover, plant phenotypic changes in response to herbivore attack on leaves or roots involve changes in many plant traits as part of the plant's inducible defence response, and this includes changes in flower traits (Barber and Gorden 2015, Lucas-Barbosa 2016, Jacobsen and Raguso 2018). Such herbivore-induced changes in flower traits can alter the attraction of flower antagonists and mutualists, resulting in plant-mediated herbivore - flower-visitor interactions. The outcome of plant-mediated herbivore - flower-visitor interactions can vary widely: herbivores can have negative, positive, or neutral effects on the visitation and performance of mutualist and antagonist flower visitors (Kessler and Halitschke 2009, McArt et al. 2013, McCall et al. 2018). This drove me to ask how specific interactions between herbivores and flower visitors are, and if specificity in plant responses to herbivory extend to flower-trait expression. Because both antagonists and mutualists influence the reproductive output of the plant (Herrera et al. 2002, Grass et al. 2018),

altered plant interactions with antagonists and mutualists due to herbivory can have consequences for plant reproduction (Lucas-Barbosa 2016, Poelman and Kessler 2016). Knowledge on the importance of indirect interactions for the assembly of flower-visitor networks on plants and associated consequences for plant fitness is limited to date.

The aim of this thesis project was to investigate how attack by a range of herbivore species affects plant interactions with mutualistic and antagonistic flower visitors and whether these interactions have associated plant fitness consequences. I was especially interested in specificity of plant-mediated herbivore - flower-visitor interactions. I used ten different herbivore species with different feeding behaviours: Some choose to feed among their favourite tissues, others are highly specialized and engage in intimate and manipulative feeding relations with the plant, whereas a few take bites or sips from different plant parts. To reveal the underlying mechanisms, I assessed specificity of herbivore-induced changes in multiple flower traits.

Study system

The black mustard, Brassica nigra

Black mustard, *Brassica nigra* L., is an annual plant belonging to the cabbage and mustards family (Brassicaceae). Plants grow often in high-density patches on open river banks and floodplains, and as early successional species in disturbed areas. Black mustard is a fast-growing species, with highly branched thick stems, and hairy blue-green leaves. This species is considered to be an obligate outcrossing species (Conner and Neumeier 1995), with a generalized pollination system. Plants flower for several weeks in which hundreds of small yellow flowers with four petals are produced. New flowers open daily, with a relatively short longevity of three to five days. Flowers are hermaphroditic, *i.e.* contain both male and female structures. Both leaves and flowers contain glucosinolates, the most characteristic secondary compounds for the Brassicaceae, and phenolics, which include important toxins, volatiles compounds, and pigments (Smallegange et al. 2007, Lucas-Barbosa et al. 2016). These compounds are involved in the defence against herbivores and in the attraction of pollinators (Hopkins et al. 2009, Borghi et al. 2017). Black mustard plants readily respond to herbivore attack with phenotypic changes including changes in foliar and floral morphological and chemical traits (Smallegange et al. 2007, Broekgaarden et al. 2011, Bruinsma et al. 2014, Lucas-Barbosa et al. 2016).

The Black mustard offers an excellent model system to investigate how attack by a range of herbivore species affects plant interactions with mutualistic and antagonistic

flower visitors and whether these interactions impact plant fitness. The plant's defence regulatory network of genes, phytohormones and metabolites involved in responses to insect herbivores has been well described (Broekgaarden et al. 2007, Broekgaarden et al. 2008, Broekgaarden et al. 2011, Stam et al. 2014), and methods to quantify these plant physiological responses to herbivory have been well developed (Broekgaarden et al. 2011, Bruinsma et al. 2014). For this plant species, flower-trait expression in response to a leaf- and flower-chewing herbivore (*Pieris brassicae* caterpillars) has been characterized. Studies with *P. brassicae* caterpillars showed that *B. nigra* responds to chewing herbivory with changes in flower traits including volatiles, nectar quantity and quality, and petal chemical composition (Smallegange et al. 2007, Bruinsma et al. 2014, Lucas-Barbosa et al. 2016). Moreover, the insect community of herbivores and pollinators of *B. nigra* has been studied in relation to herbivore-induced plant responses (Poelman et al. 2008, Poelman et al. 2010, Lucas-Barbosa et al. 2013, Pashalidou et al. 2015, Stam et al. 2018), and fitness consequences of herbivore attack have been assessed to some extent (Lucas-Barbosa et al. 2013, Pashalidou et al. 2015). Importantly, many of the insect species naturally occurring on *B. nigra* are cultured at the Laboratory of Entomology, Wageningen University, allowing for controlled induction experiments with different herbivore species.

A diverse herbivore community

Black mustard plants are colonized by a diverse herbivore community, comprising more than 30 species, of which most are specialist herbivores. Herbivore species span a variety of different feeding guilds - chewing, phloem feeding, cell piercing, mining, galling - and feeding sites at which they attack the plant - roots, leaves, buds, and flowers. In my research, I used ten herbivore species (Fig. 1). Based on shared properties in feeding guild and feeding site, the herbivore species were divided into three herbivore functional groups (HFGs): chewing herbivores (*Athalia rosae*, *Mamestra brassicae*, *Pieris brassicae* or *Plutella xylostella*), sap-feeding herbivores (*Brevicoryne brassicae*, *Myzus persicae*, or *Lipaphis erysimi*), and root-feeding herbivores (*Delia radicum*, *Heterodera schachtii*, or *Pratylenchus penetrans*). Six of these herbivores - *A. rosae, P. brassicae, P. xylostella, B. brassicae, L. eryimi, D. radicum* - are specialists on plants of the Brassicaceae family. The other four - *M. brassicae, M. persicae, H. schachtii, P. penetrans* - are generalist herbivores, occasionally feeding on brassicaceous plants. Most of these herbivores can induce systemic plant responses in brassicaceous plants (Van Dam et al. 2005, van Dam and Raaijmakers 2006, Hofmann et al. 2010, Ali and Agrawal 2012, Pareja et al. 2012).

Chewing herbivores

The selected chewing herbivores feed on aboveground tissues and display a variety of

feeding behaviours. The dark green/blackish caterpillar-like larvae of the Turnip sawfly, *A. rosae* L. (Hymenoptera: Tenthredinidae) start feeding on the leaf in which multiple eggs are laid. After the leaf is completely consumed, the larvae start to migrate to other leaves, ultimately feeding more or less solitarily. Occasionally the larvae move to the inflorescences to feed on buds and flowers (Bandeili and Müller 2010). Caterpillars of the Cabbage moth, *M. brassicae* L. (Lepidoptera: Noctuidae), are grayish-brown to green, and can be found feeding together on the leaf on which an egg clutch has been laid. Before the leaf is completely consumed, larvae disperse to other leaves and later instars can often be found solitarily on the lower, less defended, leaves of the plant. In contrast, larvae of the Large cabbage white, *P. brassicae* L. (Lepidoptera: Pieridae), are gregarious and after they start feeding on leaves, second-instar larvae move together to feed on buds and flowers. Larvae disperse at the end of their development and feed solitarily during the phase they need more food (Lucas-Barbosa et al. 2013). Green caterpillars of the Diamondback moth, *P. xylostella* L. (Lepidoptera: Plutellidae), start as miners; first instar larvae feed inside the leaf tissue. Each mine contains an individual tiny caterpillar. Later instars can often be found feeding on buds and flowers (personal observation). Chewing herbivores evolved different ways of dealing with the characteristic defensive compounds of *B. nigra*. Larvae of *A. rosae* sequester glucosinolates, and caterpillars of *P. brassicae* and *P. xylostella* have specific detoxification strategies for these compounds, whereas *M. brassicae* has a more general detoxification mechanism (Winde and Wittstock 2011, Jeschke et al. 2016).

Sap-feeding herbivores

The three aphid species (Hemiptera: Aphididae) can feed on any aboveground plant tissues, from old leaves and stems, to flowers and maturing siliques (Rusman et al. chapter 6). They are distinguishable by colour; the Cabbage aphid (*B. brassicae* L.) is white due to a waxy layer that they produce, the Mustard/Turnip aphid (*L. erysimi* Kaltenbach) is dark-green/black, and the Green peach aphid (*M. persicae* Sulzer) is light green. Winged aphids of all three species have a clear preference for inflorescences over vegetative tissues, whereas species seem to prefer to feed on different organs within the inflorescences and vegetative tissues (Rusman et al. chapter 6). The aphids vary in their way of dealing with glucosinolates: *Brevicoryne brassicae* and *L. erysimi* sequester glucosinolates (Bridges et al. 2002), whereas *M. persicae* detoxifies the compounds (Francis et al. 2005).

Root-feeding herbivores

The root-feeding herbivores include an insect species and two nematode species. Creamy-white larvae of the Cabbage root fly, *D. radicum* L. (Diptera: Anthomyiidae)

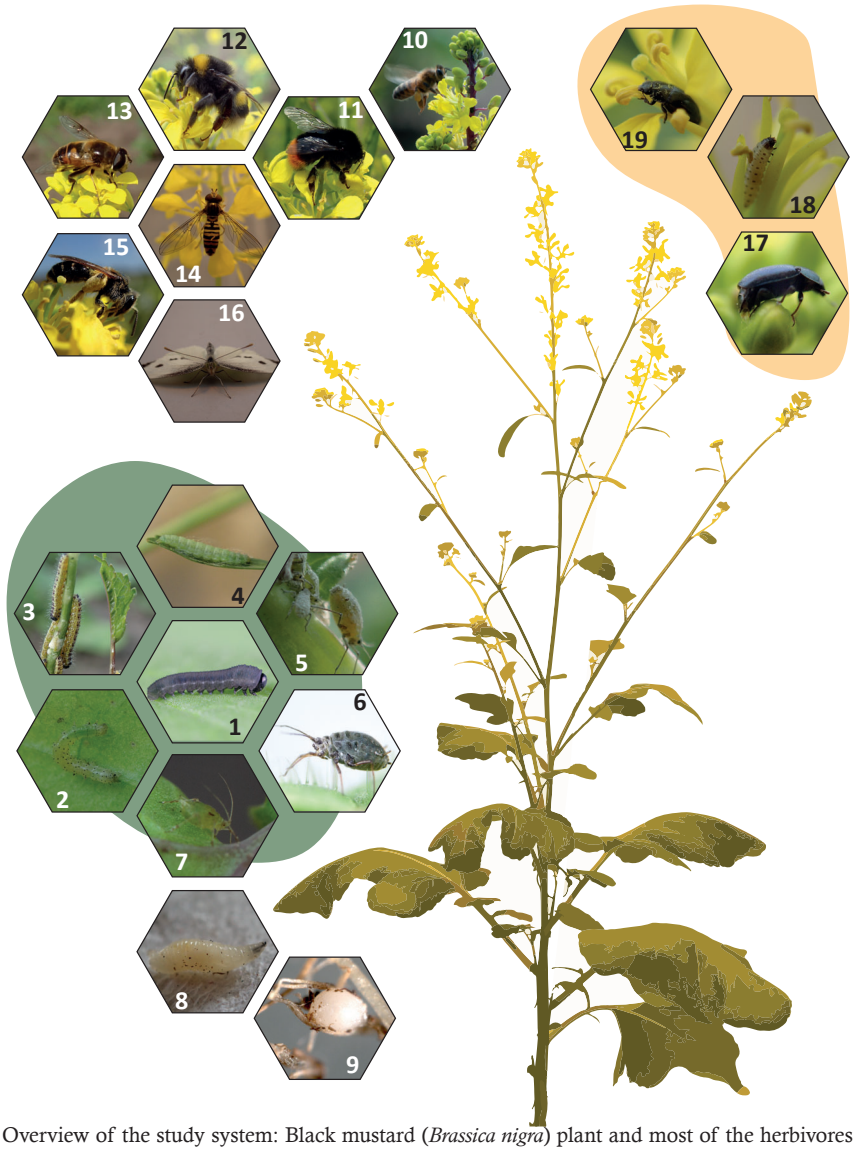

Fig. 1 Overview of the study system: Black mustard (*Brassica nigra*) plant and most of the herbivores used to experimentally infest plants in this study (1-9), along with part of the flower-associated community on which effects of herbivore-infestation were measured (10-19). Herbivores can be divided in three functional groups: chewing herbivores (*Athalia rosae* (1), *Mamestra brassicae* (2), *Pieris brassicae* (3), *Plutella xylostella* (4)), sap-feeding herbivores (*Brevicoryne brassicae* (5), *Lipaphis erysimi* (6), and *Myzus persicae* (7)), and root-feeding herbivores (*Delia radicum* (8) and *Heterodera schachtii* (9)). Flowers of black mustard are most visited by the honeybee *Apis mellifera* (10), bumblebees (*Bombus lapidarius* (11), *Bombus terrestris* (12), and other *Bombus* spp.), syrphid flies (*Eristalis* spp. (13) and several other syrphid species such as *Episyrphus balteatus* (14)), solitary bees (15), and butterflies (*Pieris* spp. such as *P. brassicae* (16), and other Lepidoptera). Among the most abundant florivores are pollen beetles, *Meligethes* spp. (pollen beetle female laying an egg (17), second instar pollen beetle larvae (18), pollen beetle adult resting amidst the anthers of a black mustard flower (19)). Photograph credits: Jitte Groothuis, Dani Lucas-Barbosa, and Quint Rusman.

feed mainly on the tap root of *B. nigra.* They chew tissue from the root surface and mine into the root. Plant-parasitic nematodes are tiny roundworms of about 1 mm in length that can be found at densities up to 30 million/m^2 in the soil (Norton and Niblack 1991). Lifecycles of most plant parasitic nematodes are relatively simple with an eggs stage, four juvenile stages, and an adult stage. Juveniles of the Sugar beet cyst nematode, *H. schachtii* (Tylenchida: Heteroderidae), invade the roots and induce the formation of a syncytium, which functions as feeding site for this endoparasitic nematode (Turner and Rowe 2006). The Root lesion nematode, *P. penetrans* (Tylenchida: Pratylenchidae) is a migratory endoparasitic nematode (Van Dam et al. 2005). Juveniles and adults enter the root cortex using their stylet, feed on cells within the roots until the cell lyses, and then move on to find new healthy cells. It is not known how these root herbivores deal with glucosinolates typical of the Brassicaceae.

Mutualistic flower visitors

Brassica nigra is visited by a broad range of flower visitors including bees, flies, butterflies, beetles, wasps, sawflies, aphids, true bugs, and caterpillars. The mutualistic pollinator community consists of six major groups of pollinators (Fig. 1): honeybees (*Apis mellifera*), bumblebees (*Bombus terrestris, Bombus lapidarius, Bombus pratorum*, *Bombus hortorum*, and other *Bombus* spp.), syrphid flies (several *Eristalis* spp. and several other syrphid species), solitary bees (several *Andrena* and *Lasioglossum* species but also other Apidae excluding *Bombus* spp.), other flies (non-syrphid Diptera), and butterflies (*Pieris* spp. and other Lepidoptera). I assume that most of these insects contribute to pollination, although this has not been investigated for *B. nigra* so far. The behaviour of two pollinator species, the butterfly *P. brassicae* and the syrphid fly *Episyrphus balteatus,* was investigated in the greenhouse. Butterflies mainly feed on nectar, and not pollen, while feeding on *B. nigra* plants. Compared to other pollinators, *P. brassicae* has a low visitation frequency in the field (LucasBarbosa et al. 2013). Yet, this butterfly may be important for long-distance pollen dispersal (Courtney et al. 1982). Syrphid flies can feed on both nectar and pollen, but mainly feed on pollen of *B. nigra*. *Episyrphus balteatus* is a common flower visitor and efficient pollinator of plants in the Brassicaceae family (Jauker and Wolters 2008).

Floral antagonistic pollen beetles

Pollen beetles, *Meligethes* spp. (Coleoptera, Nitidulidae) are among the most abundant floral antagonistic species of *B. nigra*. Populations of these beetles consist of about 90% of *M. aeneus* (Fabricius) and the remainder is likely *M. viridescens* (Fabricius) (Billqvist and Ekbom 2001). In early spring, adult beetles emerge from their overwintering sites and feed on pollen of plants from many different plant families. *Meligethes viridescens* appears

later in the season than *M. aeneus*, because *M. viridenscens* needs higher temperatures to emerge from overwintering sites (Metspalu et al. 2011). Mating exclusively happens on cruciferous plants, and eggs are laid inside flower buds. Adults find brassicaceous plants using colour and odour cues (Cook et al. 2002, Jönsson et al. 2007, Doering et al. 2012). Females prefer to lay eggs in buds between 2 and 3 mm in size, and in general one to three eggs are inserted (Ekbom and Borg 1996). After a few days the beetle larvae hatch from eggs, and the creamy-white larvae with a brown-black head start feeding on the tissues within the bud, mostly the anthers. When the infested bud opens, the larva moults to the second instar, and moves around the plant to feed on pollen from various flowers. At maturity, about 20 to 30 days after hatching, the larvae drop to the ground and pupate in the soil (Metspalu et al. 2011). The adults emerge about a month later, in late summer/early autumn, and feed on pollen of plants from many different plant families before overwintering.

Outline of this thesis

Chapter 2 addresses the current knowledge on flower plasticity in response to herbivory and places flower plasticity in a community context. The chapter reviews the extent to which herbivore-induced plant responses affect multiple flower traits, and the underlying molecular mechanisms in floral plasticity. I discuss which flower traits often mediate interactions between herbivores and other flower-associated organisms, and if such herbivore - flower-visitor interactions are functional for the plant. Considering the ecological consequences of flower plasticity for flower-associated organisms, I argue that the ecology of plastic flowers can best be understood within a community context. However, specificity in plant-mediated herbivore - flower-visitor interactions and consequences for flower-visitor community assembly and plant reproduction is poorly understood to date.

The main goal of **Chapter 3** was to evaluate specificity of plant responses to different herbivore species and how these plant responses affect flower visitors and plant fitness. In the field, flowering plants were exposed to one of ten herbivore species, including chewing herbivores, sap-feeding herbivores, and root herbivores. The effects on pollinator visitation, pollen beetle (*Meligethes* spp.) abundance and performance, and plant seed production were assessed.

Plant responses to herbivores are not only determined by the herbivore, but can vary with plant ontogeny. Plants are considered to display ontogenetic variation in growth, defence, and reproduction to maximize reproduction. In **Chapter 4**, my aim was to investigate whether herbivore - flower-visitor interactions and associated plant fitness consequences are different when herbivore attack varies over plant ontogeny. Plants were

exposed to different herbivore species at three plant ontogenetic stages (vegetative, bud, flowering). Effects of plant ontogeny and herbivore identity on plant flowering traits, interactions with flower visitors, and plant reproduction were measured.

Chapter 5 presents research on the mechanisms through which different herbivores affect pollinator behaviour. I was interested in the specificity of herbivore-induced changes in flower traits, and whether changes in specific traits can predict the effects on pollinator behaviour. I recorded changes in a number of flower traits, such as flower colour, volatile emission, and rewards, and the behaviour of two pollinating insects upon exposure of plants to different herbivore species.

Specificity of herbivore-induced changes in flower traits may go beyond interspecific variation in herbivore feeding behaviour. When plants start flowering, some herbivores move to feed on the flowers, while others remain feeding on leaves. Herbivore choice for leaves or flowers may lead to differences in inducible plant responses including flower traits and may affect pollinator visitation. In **Chapter 6**, I use manipulative experiments to explore whether herbivore choice for a given feeding site determines the outcome of plant-mediated herbivore-pollinator interactions. Three herbivore species were each placed on either leaves or flowers of flowering *B. nigra* plants and the responses of two pollinator species visiting flowers were recorded. I measured the preference of the herbivores for vegetative and floral tissues and their performance on both tissues. The results are placed in a broad eco-evolutionary framework by discussing trait variation in antagonist-mutualist interactions and the consequences for eco-evolutionary dynamics.

In **Chapter 7**, I integrate the findings of my thesis. Herbivore - flower-visitors interactions are placed in an eco-evolutionary framework, and I emphasize the commonness of such interactions and the importance of trait variation of the various players involved. To assess the importance of herbivory for plant evolution, it is necessary to extend our knowledge on the consequences of herbivory beyond plant seed production, and elucidate the contribution of direct effects of herbivory and indirect effects *via* other community members. Herbivore - flower-visitor interactions provide an excellent system to assess the importance of indirect interactions in eco-evolutionary dynamics. The findings presented in this thesis provide evidence that indirect interactions between mutualists and antagonists are common in plant-associated communities, and likely affect eco-evolutionary dynamics of plants and insects.

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Chapter 2

The ecology of plastic flowers

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Submitted

Abstract

Plant phenotypic plasticity in response to herbivore attack includes changes in flower traits. Such herbivore-induced changes in flower traits have consequences for interactions with flower visitors. Here, we synthesize current knowledge on the specificity of herbivore-induced changes in flower traits, the underlying molecular mechanisms and the ecological consequences for flower-associated communities. Herbivore-induced changes in flower traits seem to be to a large extent herbivore-species-specific, mediated by tissue-specific gene expression and regulatory components. The extensive plasticity of flowers results in a highly connected web of interactions within the complete flower-associated community. We argue that the adaptive value of herbivore-induced plant responses and flower plasticity can only be fully understood from a community perspective, rather than from pairwise interactions.

Keywords: flower visitors, flower traits, herbivore-induced plant responses, plant defence, phenotypic plasticity, specificity

Flowers and their environment

The angiosperms, which comprise the majority of plant species, are characterized by an incredible diversity in flowers that range from the tiny millimetre-long flowers of duckweed (*Lemna minor*) to the giant meter-wide corpse flower (*Rafflesia arnoldi*) (Soltis and Soltis 2014, Moyroud and Glover 2017). The diversity of shapes, colours and scents of flowers is largely a result of evolution with an even more diverse flower-associated community (Strauss and Whittall 2006, Schiestl and Johnson 2013). This community includes mutualists, such as **pollinators** (see Glossary), predators and parasitoids, as well as antagonists, such as herbivores and pathogens (McCall and Irwin 2006, Lucas-Barbosa et al. 2011, Junker and Keller 2015). As flowers are the reproductive organs of the plant, their displays are under selection to maximize reproduction under varying environmental conditions. Flowering plants are therefore expected to respond to the environment, including visitation by antagonists and mutualists. Indeed, flower traits readily change in response to herbivory or pathogen attack, even when these antagonists attack plant organs other than flowers. Flower traits also change in response to visitation by mutualists such as pollinators (van Doorn 1997, Willmer et al. 2009, Rodriguez-Saona et al. 2011, Lucas-Barbosa et al. 2016), and beneficial microbes (Lenaerts et al. 2017, Rering et al. 2018, Sobhy et al. 2018). Here, we focus on herbivore-induced changes in flower traits, because despite accumulating data on this topic, we still have important knowledge gaps. For instance, it is unclear if there are general patterns in flower **phenotypic plasticity** to different types of herbivores, which changes in flower traits are **functional** for the plant, and which traits merely change as pleiotropic effects of herbivore-induced plant responses. Because changes in flower traits mediate interactions with multiple flower-associated community members, we need to adopt a community approach to understand flower trait plasticity (McCall and Irwin 2006, Lucas-Barbosa et al. 2011, Lucas-Barbosa 2016, Jacobsen and Raguso 2018). In this review, we discuss 1) the current knowledge on the **specificity of induction** and to what extent herbivore-induced changes affect flower traits, 2) what are the underlying molecular mechanisms of flower plasticity, 3) functionality of flower plasticity in relation to which flower traits often mediate interactions between herbivores and other flower-associated organisms, and 4) the ecological consequences of floral plasticity for flower-associated communities.

Specificity of herbivore-induced floral plasticity

The influence of herbivore-induced changes on flower traits and consequences for flower visitors was first recognized more than two decades ago (Strauss et al. 1996) (Fig. 1). Since then, it has become apparent that floral plasticity in response to herbivory differs among plant species, and ranges from limited to extensive plasticity (Burkle and Runyon 2016, Hoffmeister et al. 2016, Lucas-Barbosa 2016) (Table 1). For example,

folivory by *Trichoplusia ni* affects flower morphology of *Campanula rotundifolia*, floral volatile blend composition of *Heterotheca villosa*, whereas it does not affect flower traits of *Phacelia hastata* or *Potentilla recta* (Burkle and Runyon 2016). In *Brassica nigra*, a range of herbivore species that commonly attacks this species can change multiple flower traits simultaneously, including floral morphology, volatiles, colour, nectar and pollen production (Rusman et al. 2019 - chapter 5). Some flower traits are more plastic than others, either because their expression is more plastic (Rozendaal et al. 2006, Siebenkäs et al. 2015), or because they are more closely connected to physiological regulation of plant defences (Box 1). Especially flower phenology, morphology, and volatile emission seem to be plastic in response to herbivory, whereas nectar production changes in some cases, but not in others (Table 1). The extent of plasticity of flower colour and pollen production and chemistry is difficult to assess because these traits are rarely investigated, probably because of difficulties to do so accurately. When measured, colour and pollen production show changes in response to herbivory, but not necessarily (Poveda et al. 2005, Schiestl et al. 2014, Lucas-Barbosa et al. 2016, Rusman et al. 2019 - chapter 5). For instance, the reflectance spectrum of flowers may not change in response to herbivory (Schiestl et al. 2014, Hoffmeister et al. 2016), or change by reflecting higher or lower intensities of specific wavelengths like yellow and UV (Rusman et al. 2019 - chapter 5). When pollen production changes in response to herbivory, this is mostly by a reduction in the amount of pollen produced (Poveda et al. 2005, Rusman et al. 2019 - chapter 5).

Glossary

Adaptive: enhancing fitness, i.e. the contribution of an individual to the gene pool of the next generation
Autogamous selfing: Self-pollination within a flower
Diffuse selection: Natural selection imposed on a community member is dependent on the presence or absence of other species in the community (Strauss et al. 2005)
Folivores: Consumers of aboveground vegetative tissues.
Florivores: Consumers of developing flower buds or mature flowers before the development of the seed coat and including consumers of bracts, sepals, petals, stamens, pistils, pollen and ovules (McCall and Irwin 2006). Also includes sap-feeding consumers that feed from floral stalks.
Functional: Same as *adaptive*.
Nectar thief: flower visitor that collects nectar without damaging flowers, but does not contribute to pollination (Inouye 1980).
Nectar robber: flower visitor that damages flowers while collecting nectar, but does not contribute to pollination (Inouye 1980).
Pollinator: Flower visitor that contributes to successful pollination, *i.e.* to the transfer of pollen from one flower to another conspecific flower.
Plant ontogeny: Development of a plant from seed to mature seed-producing plant (Barton and Boege 2017).
Phenotypic plasticity: The capacity of a single genotype to display different phenotypes in response to different environments (Whitman and Agrawal 2009).
Specificity of induction: differential changes in phenotype in response to different inducers.

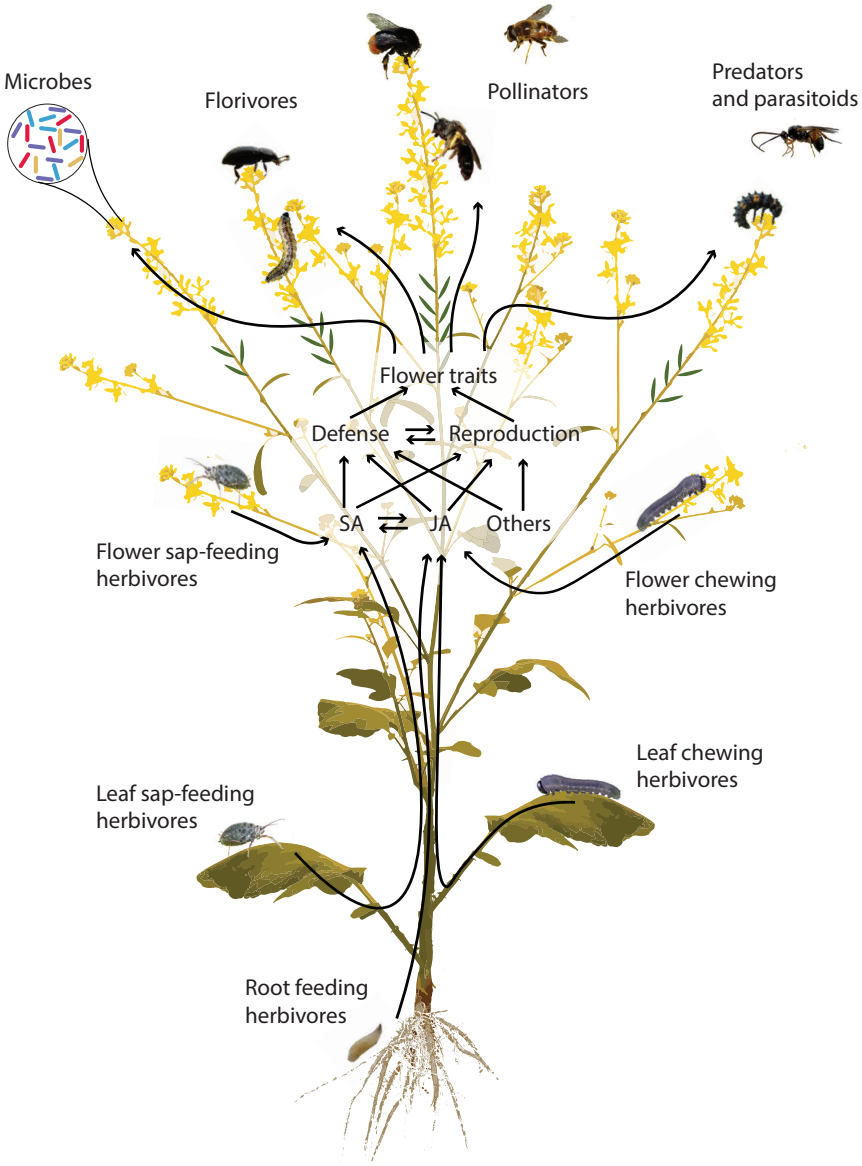

Fig. 1 Illustration of the potential effects of herbivore-induced plant responses on flower traits and consequences for flower-associated organisms. Plant responses to herbivory involve the systemic activation of phytohormonal signalling pathways. Overall patterns illustrate that above- and belowground chewing herbivores induce the jasmonic acid (JA) pathway, whereas sap-feeding herbivores induce the salicylic acid (SA) pathway. Both defence and reproduction are mediated by phytohormones such as JA and SA, but also others. Moreover, defence and reproduction are physiologically linked *via* various mechanisms. Both defence- and reproduction-related processes affect the expression of flower traits. Flower traits mediate interactions with flower-associated community members such as floral microbes, florivores (including seed predators, and nectar or pollen thieves and robbers), pollinators, and predators and parasitoids. Photograph credits: Jitte Groothuis, Dani Lucas-Barbosa, Erik Poelman, and Quint Rusman.

Table 1. Phenotypic plasticity of flowers in response to herbivory with its effects on subsequent flower visitation by other organisms. Compared to uninfested plants, herbivory may increase (+), decrease (-), or have neutral (O) effects on plant traits and flower visitors, or herbivory may change traits in a specific direction (C). No entry indicates that the trait was not assessed for the respective herbivore. Question mark indicates that no exact data was presented or accessible, or no proper statistical analysis was done. We only included studies that assessed 3 or more trait groups. Plant species: Bn = *Brassica nigra*, , Br = *B. rapa*, Pa = *Palicourea angustifolia*, Sa = *Sinapis arvensis*, Sl = *Silene latifolia*, Cr = *Campanula rotundifolia*, Hv = *Heterotheca villosa*, Ph = *Phacelia hastata*, Pr = *Potentilla recta*, Mg = *Mimulus guttatus*, Sp = *Solanum peruvianum*, Rr = *Raphanus raphanistrum*, Ps = *Pastinaca sativa*. References: 1 = Rusman et al. 2019 - chapter 5, 2 = Zangerl and Berenbaum 2009, 3 = Kessler and Halitschke 2009, 4 = Hoffmeister et al. 2016, 5 = Schiestl et al. 2014, 6 = Lucas-Barbosa et al. 2016, 7 = Bruinsma et al. 2014, 8 = Strauss et al. 1996, 9 = Poveda et al. 2005, 10 = Cozzolino et al. 2015, 11 = Burkle and Runyon 2016, 12 = Chautá et al. 2017, 13 = Ivey and Carr 2005.

					Flower morphology								**Flower volatiles**		**Flower colour**			**Petal quality**		**Flower rewards**					**Behavioural responses**						
Feeding guild or site	Insect herbivore	Plant species	Flower abundance	Flower phenology	Petal/Corolla size	Petal/Corolla length	Petal/Corolla width	Petal roundness	Flower display size	Anther Length	Style Length	Herkogamy	Total volatile emission	Volatile blend	Reflectance spectrum	Yellow reflectance	UV reflectance	Defensive chemistry	Nutritional chemistry	Nectar volume	Nectar sugar conc.	Nectar chemistry	Pollen grains	Pollen chemistry	Syrphid fly behaviour	Butterfly behaviour	Hummingbird behaviour	Bee behaviour	Predators and parasitoids	Florivores	References
	Athalia rosae	Bn	O		O	_	O	_	O				O	C	C	_	O			_					O	O					1
	Depressaria pastinacella	Ps	_						_				+	C											_						2
	Manduca sexta	Sp	O						O	O	_	_		C														_		_	3
	Phaedon cochleariae	Sa	O	+ O			+ O		O				O ?	O ?																	4
	Pieris brassicae	Br		+					O				_	?	O			O										_	+		5
	Pieris brassicae	Bn												C	O	O	O	C							_	O					6

Chewing herbivores	*Pieris brassicae*	Bn												C						O	C	O			_	_		_		7
	Pieris rapae	Rr	O	_			_	_												_			_		_			_		8
	Plutella xylostella	Bn	O			O	O	+	_	+			O	C	C	O	O								O	_				1
	Spodoptera littoralis	Br		_						O			_	?	O			+										_		5
	Spodoptera littoralis	Sa	O	_		_					_									O										9
	Spodoptera littoralis	Sl	O				O	O					? +	?						O	O									10
	Trichoplusia ni	Cr	O							_			O	O														O		11
	Trichoplusia ni	Hv	O							O			O	C														_		11
	Trichoplusia ni	Ph	O							O			O	O														O		11
	Trichoplusia ni	Pr	O							O			O	O														O		11
	Zeromastax selenesii	Pa					O				O	_	O	O						_	+						_	O		12
Sap-feeding herbivores	*Brevicoryne brassicae*	Bn	O			O	_	O	_	O			O	C	C	O	+			_					_	_				1
	Lipaphis erysimi	Bn	O			+	O	+	_	O			O	C	C	O	+			O			O		+	+				1
	Philaenus spumarius	Mg	O			_	_	O		O		+													O			O		13
Root herbivores	*Agriotes* sp.	Sa	O	O		O					O									O										9
	Delia radicum	Bn	O			+	O	+	_	O			O	C	C	O	O			O			_		+	O				1

Besides variation in plant species and traits, herbivore-induced changes in flower traits vary with herbivore identity and feeding behaviour (Table 1). Because herbivore-induced plant responses often contain a systemic component, and defence and reproduction are physiologically linked (Box 1), we expect similar patterns of specificity in the induction of flower traits as for defence traits. Specificity of induction of foliar defence traits often includes a general component based on the feeding mode and feeding site of the herbivore and a more specific component based, among others, on herbivore identity (Ali and Agrawal 2012, Erb et al. 2012, Thaler et al. 2012, Johnson et al. 2016). The limited direct evidence we have suggests that changes in flower traits are to a large extent herbivore-species specific (Table 1). Indirect evidence gained by comparing different studies supports this. For example, the application of jasmonic acid (JA) on leaves of *Brassica nigra*, which induces the general component of herbivore-induced plant responses against chewing herbivores, reduced nectar production in the flowers, whereas folivory by *Pieris rapae* or *P. brassicae* caterpillars, two chewing herbivores that induce JA, increased nectar production or had no effect (Bruinsma et al. 2008, Bruinsma et al. 2014). To explain differences in specificity of induction between foliar and floral tissues, we need to consider the underlying physiological mechanisms.

Box 1: Physiological links between defence and reproduction

Plant defence and reproduction appear to be linked because defensive and reproductive traits are correlated (Adler et al. 2006, Irwin and Adler 2006) and the expression of flower traits changes in response to herbivore attack. Various mechanisms have been proposed to explain these links (Lucas-Barbosa 2016, Jacobsen and Raguso 2018). All traits share resources from the limited nutrient pool of the plant and reallocation of resources to defence can impair reproduction (Herms and Mattson 1992). Defence and reproductive traits share phytohormonal signalling pathways including e.g. jasmonic acid (JA) and salicylic acid (SA) (Avanci et al. 2010, Rivas-San Vicente and Plasencia 2011, Erb et al. 2012, Thaler et al. 2012, Yuan and Zhang 2015), and herbivore-induced increases or decreases in any of these phytohormones potentially alter the expression of flower traits (Yuan and Zhang 2015). Downstream of phytohormonal signalling, the expression of both flower and defence traits is controlled by genetic and biochemical pathways. Genetic pleiotropy *via* gene regulatory networks and shared transcription factors, or individual genes involved in multiple regulatory pathways can connect defence and reproduction (Hemm et al. 2003, Nagpal et al. 2005, Kaufmann et al. 2009, Pajoro et al. 2014, Rasmann et al. 2018, van Es et al. 2018). Biochemical pleiotropy can occur *via* changes in pools of shared precursors or enzymes (Fineblum and Rausher 1997, Muhlemann et al. 2014, Johnson et al. 2015, Junker et al. 2018). Other physiological constraints can lead to co-expression of defence and reproductive traits, such as the passive diffusion of defensive metabolites from the phloem to flower organs (Adler 2000, Jacobsen and Raguso 2018), or herbivory-induced changes in the chemical environment of the cell, which are important for phytohormonal signalling (Erb et al. 2012), but can also change the redox state of pigments, leading to a shift in light absorbance (Borghi et al. 2017). Finally, individual traits can have multiple functions and be involved in defence and reproduction (Gronquist et al. 2001, Schiestl et al. 2014). For example, flowering plants use floral volatiles to attract pollinators, but also natural enemies of herbivores (Schiestl et al. 2014). Redirection of flower traits to a defensive function upon herbivore attack with associated changes can render these traits sub-optimal for reproductive functions.

Physiological regulation of flower plasticity

Herbivore-induced changes in plant leaves are well known to be mediated by phytohormonal signal-transduction pathways (Pieterse et al. 2012). Emerging evidence indicates that flowers, just as leaves, respond to herbivory by activating phytohormonal signalling. Increased expression of the JA biosynthesis gene *LIPOXYGENASE-2* (*LOX2*) can be induced in various floral tissues by exogenous methyl jasmonate (Jensen et al. 2002), and leaf herbivory can induce the expression of JA biosynthesis genes, such as *ALLENE OXIDE SYNTHASE* (*AOS*) in the flowers (Zhou et al. 2017, Peng et al. 2018), with subsequent induction of JA (McArt et al. 2013, Zhou et al. 2017, Chrétien et al. 2018). In contrast to leaves, concentrations of floral salicylic acid (SA) have so far not been shown to change in response to herbivory, for abscisic acid (ABA) evidence suggests that herbivory has either no effect or results in increased concentrations of ABA in flowers (Zhou et al. 2017, Chrétien et al. 2018). Despite the apparently conserved phytohormonal signalling in leaves and flowers (Box 2), important tissue-specific patterns in the accumulation and regulation of signalling pathway components have been identified (Schmid et al. 2005). This may explain the discrepancy in specificity of herbivore induction between foliar and floral tissues. So far, the accumulation and regulation of foliar and floral tissue-specific components have mostly been documented for the JA-pathway. The constitutive accumulation of ALLENE OXIDE CYCLASE (AOC) mRNA and proteins was found to be higher in tomato flowers compared to leaves, and also differed between flower tissues (Hause et al. 2000). The accumulation of AOC mRNA and proteins was accompanied by tissue-specific increases in the concentrations of JA, 12-oxo-phytodienoic acid (OPDA), and jasmonoyl-L-isoleucine (JA-Ile). When *AOC* was overexpressed in tomato, this increased JA and OPDA concentrations in buds and flowers, but not in leaves (Miersch et al. 2004). The increase in JA and OPDA concentrations differed among floral tissues, resulting in specific ratios of these compounds in various flower organs. Biosynthesis of JA in the stamens of *A. thaliana* starts with the expression of *DEFECTIVE IN ANTHER DEHISCENCE1*, which is not expressed in other flower tissues (Ishiguro et al. 2001). In pea (*Pisum sativum*), a *LOX* gene has been identified which is predominantly expressed in the flowers, and shows differential expression in flower tissues, with the highest expression in petals and carpels (Rodríguez-Concepción and Beltrán 1995). Together, these results suggest differential regulation of JA biosynthesis between leaves and flowers, and even different flower organs, *via* the tissue-specific accumulation of conserved pathway components and the presence of unique pathway components. Such tissue specificity is also present in the JA-induced part of the signalling pathway. Specific types of JASMONATE ZIM-DOMAIN (JAZ) and MYB proteins are found in flowers, that are not expressed in leaves (Reeves et al. 2012, Stitz et al. 2014, Sherif et al. 2015, Li et al. 2017). These specific JAZ proteins are important for the accumulation of constitutive defences (Li et al. 2017). Moreover,

JAZ proteins that occur both in leaves and flowers can serve different functions, with different regulatory targets (Oh et al. 2013). Especially JAZ proteins and transcription factors (TFs) such as MYCs, MYBs and APs, may be important for tissue-specific regulation of particular processes, such as flower development and defence (Sherif et al. 2015, Zhai et al. 2015, Wang et al. 2017, Yu et al. 2018). For example, in *Arabidopsis* flowers JAZ1, 3, 4 and 9 proteins interact with the TFs TOE1 and TOE2 to regulate *CORONATINE INSENSITIVE 1* (*COI1*)-dependent flowering, but not *COI1*-dependent defence gene expression (Zhai et al. 2015). Also, MYB and WRKY TFs, and multiple *CYP94*-genes – important in feedback mechanisms of JA-Ile – are differentially expressed in leaves and flowers, and even in different floral tissues (Wagstaff et al. 2009, Stitz et al. 2014, Bruckhoff et al. 2016).

Regarding the SA-pathway, leaves and flowers can contain different concentrations of free and total SA, which results in tissue-specific transcriptional responses of SA-regulated defence genes (Ederli et al. 2015, Chrétien et al. 2018). Moreover, SA concentrations and gene transcription levels also differ between sepals and petals, although the differences are smaller than compared to leaves. The TF HAHB10 is induced in sunflower leaves after SA treatment and pathogen attack, but repressed after wounding (Dezar et al. 2011). This TF increases the expression of multiple genes involved in flowering, and represses the expression of multiple genes involved in JA- and SA-mediated defence. Interestingly, this TF is mostly expressed in mature leaves, and almost absent in floral tissues, except for the carpels (Dezar et al. 2011).

This suggests that regulation of development and defence is different in vegetative and floral tissues of flowering plants. Tissue-specific gene expression patterns and regulatory components likely lead to different plant responses in leaves versus flowers. Indeed, floral and foliar herbivory induce different changes in the plant (Kessler and Halitschke 2009, Peng et al. 2018), and plant responses to foliar herbivory differ for foliar and floral tissues (McArt et al. 2013, Bruinsma et al. 2014, Lucas-Barbosa et al. 2016). Flowers even respond differently to attack on different systemic tissues. For example, root and foliar herbivory induce different changes in flower traits (Barber et al. 2011, Rusman et al. 2019 - chapter 5) (Fig. 1). Moreover, the plant as an integrated phenotype can adopt different defence strategies depending on **plant ontogeny** (Barton and Boege 2017), and specifically between vegetative and flowering stages. The timing of herbivory over plant ontogeny can therefore result in different patterns of herbivore-induced plant responses (Barton and Koricheva 2010). Taken together, tissue-specific and ontogeny-specific expression of genes and regulatory components with resultant differential expression of plant responses likely explain the differences in specificity of herbivore-induced changes in plant traits between leaves and flowers. Such knowledge on the underlying mechanisms allow us to manipulate the plant phenotype, preserving the context of these complex interactions, to test their effects on other organisms present in the environment,

and their consequent functionality.

Functionality of floral plasticity

Flower traits that commonly change in response to herbivory are hypothesised to be **adaptive** by mediating interactions that maximize reproductive output, thus benefiting plant fitness (Fig. 2). By now, we acknowledge that floral volatiles commonly change in response to herbivory and that these are exploited by natural enemies of the herbivores (Dannon et al. 2010, Schiestl et al. 2014, Silveira et al. 2018). Although plant fitness benefits of the attraction of natural enemies of herbivores are intuitive and have been shown for plants in interactions with herbivores and natural enemies in the vegetative stage (Schuman et al. 2012), these remain largely elusive for flowering plants. Herbivore-induced changes can increase the attraction of pollinators (Poveda et al. 2003, Soper Gorden and Adler 2018, Rusman et al. 2019 - chapter 5), potentially *via* changes in floral volatile emission (Cozzolino et al. 2015). An increased attraction of pollinators can increase reproductive output (Cozzolino et al. 2015, Rusman et al. 2018 - chapter 3), but not necessarily (Poveda et al. 2003), and this likely depends on conditions such as pollen and resource limitations. Herbivore-induced changes can lead to increased resistance to **florivores**, seed predators, and **nectar thieves** (Soper Gorden and Adler 2016, 2018), by changes in flower chemistry (McCall and Irwin 2006, McArt et al. 2013, Li et al. 2018, McCall et al. 2018). Leaf-herbivore-induced resistance to seed predators has been shown to benefit plants of *Oenothera biennis* by reducing seed predation to a large extent, whereas leaf herbivory itself had little impact on reproductive output (McArt et al. 2013). Hence, herbivore-induced changes in flower traits that mediate pairwise interactions with mutualist or antagonist flower visitors may be adaptive for the plant.

Box 2: Defence regulation in flowers: What can we learn from leaves?
Research on flower development suggests that the gene regulatory networks for various phytohormones, and jasmonic acid (JA) in particular, is conserved in leaves and flowers (Wagstaff et al. 2009, Yuan and Zhang 2015). Flowers show expression of multiple JA biosynthesis genes and products similar as in leaves, such as *LIPOXYGENASE* (*LOX*), *ALLENE OXIDE SYNTHASE* (*AOS*), 12-oxo-phytodienoic acid (OPDA), and *OPDA REDUCTASE* (*OPR*), and produce JA locally (Sanders et al. 2000, Wagstaff et al. 2009, Reeves et al. 2012, Li et al. 2017). Later steps in JA signalling also seem to work similarly in leaves and flowers, with essential roles for CORONATINE INSENSITIVE 1 (COI1) and JASMONATE ZIM-DOMAIN (JAZ) proteins and WKRY and MYB TFs (Stitz et al. 2014, Zhai et al. 2015). In addition, this is evident from similar expression patterns in leaves and flowers for three *NAC* genes encoding JA regulatory proteins (Wagstaff et al. 2009) and some *JAZ* genes: *JAZ5* and *JAZ7* (Reeves et al. 2012, Chini et al. 2016). Thus, the backbone of phytohormone signalling, and for JA in particular, appears conserved in leaves and flowers.

In contrast to benefits, changes in flower traits may result in ecological costs (Kessler and Halitschke 2009, Poelman and Kessler 2016). The most commonly reported ecological cost is a reduction in pollinator visitation (Kessler and Halitschke 2009, Lucas-Barbosa 2016), mediated by herbivore-induced changes in floral volatiles, morphology, colour, and/or rewards (Fig. 2) (Kessler and Halitschke 2009, Schiestl et al. 2014, Chautá et al. 2017, Rusman et al. 2019 - chapter 5). Such reductions in pollinator visitation can negatively affect plant reproductive output (Chautá et al. 2017), but not necessarily, again depending on conditions such as pollen and resource limitations (Krupnick and Weis 1999, Ghyselen et al. 2016). Herbivore-induced flower traits may also interfere with the optimization of pollination. For example, plants use honest signalling to increase flower constancy and pollination effectiveness (Wright and Schiestl 2009). With honest signalling, plants provide one or a few cues, such as volatile compounds or flower size, that are reliable indicators of flower rewards. By altering flower volatile emission,

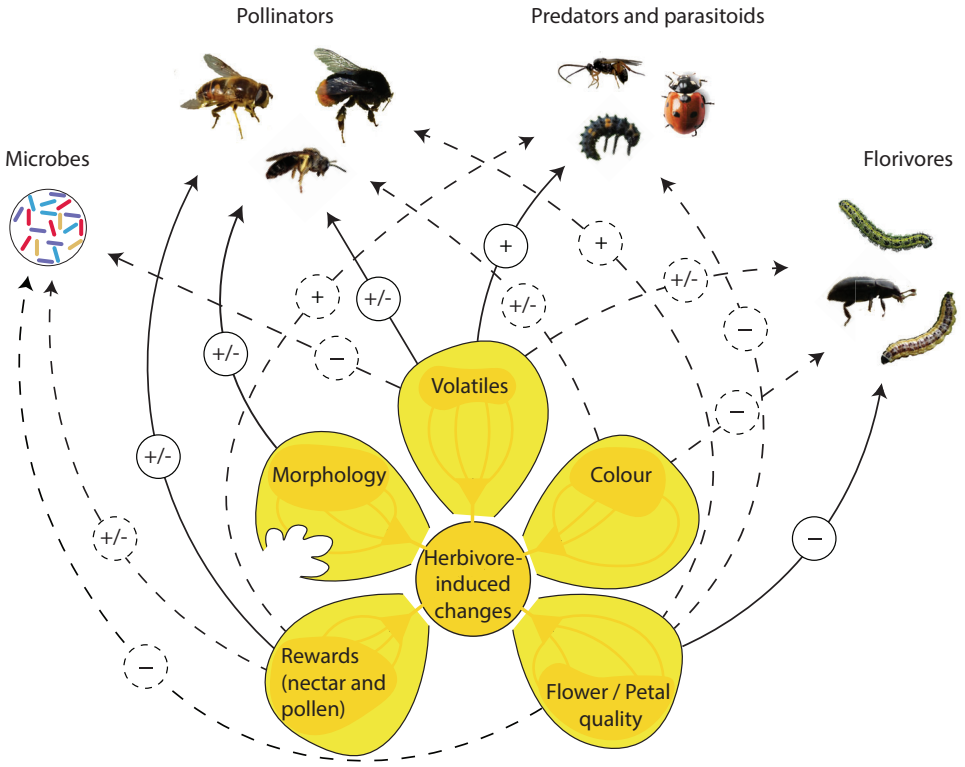

Fig. 2 Schematic representation of herbivore-induced changes in flower traits that mediate interactions with flower-associated organisms. Solid lines indicate direct evidence, dashed lines are based on indirect evidence. The sign in the circle represents the direction of effect, where + = positive effects, - = negative effects, +/- = both positive and negative effects, on the attraction, abundance, or performance of the insects and microbes. The term florivore here includes seed predators, and nectar and pollen thieves and robbers. Photograph credits: Erik Poelman and Quint Rusman.

morphology, size, and nectar and pollen production, herbivores potentially interfere with honest signalling (Junker et al. 2018, Rusman et al. 2019 - chapter 5). Herbivory can also alter local changes in flower traits in response to visitation by flower visitors. Pollination often induces changes in flower volatile emission, colour and morphology, resulting in reduced visitation by other pollinators to already pollinated flowers (van Doorn 1997, Willmer et al. 2009, Rodriguez-Saona et al. 2011, Lucas-Barbosa et al. 2016). Pollination-induced changes in, for example, floral volatiles can be dependent on whether the plant is simultaneously attacked by herbivores (Lucas-Barbosa et al. 2016), which might reduce pollination effectiveness. Interestingly, ecological costs of changes in flower phenotype in terms of reduced pollination can be compensated for by simultaneous changes in plant mating system (Johnson et al. 2015), by for example increasing **autogamous selfing** (Penet et al. 2009). Herbivore-induced changes can increase the attraction of florivores by changing apparency traits such as plant height, or flower colour and volatile emission (Soper Gorden and Adler 2016), with potential negative effects on plant reproduction (Theis and Adler 2012). Thus, herbivore-induced changes in flower traits can be detrimental for plant reproduction *via* pairwise interactions with flower visitors.

Herbivore-induced changes in pairwise interactions with flower visitors seem adaptive in some cases, such as increased resistance to florivores, maladaptive in others, such as the reduced visitation by pollinators (Fig. 2). To understand the adaptive value of flower plasticity with such contrasting differences on pairwise interactions, it is important to adopt a community perspective.

Consequences for flower-associated communities

Herbivore-induced changes in flower traits have consequences for the flower-associated community. Some flower visitors utilize flower traits for host-plant location, whereas the growth and survival of most of these organisms is affected by the chemical composition of the flowers (McCall and Irwin 2006, Dötterl and Vereecken 2010, Aleklett et al. 2014). Therefore, we expect herbivore-induced changes in flower traits to have major impact on flower-associated community dynamics. Flowers are visited by one or multiple pollinator species, predominantly bees, flies, butterflies and moths, or beetles. The composition of the pollinator community might be very specific for plant species, even when those plants are part of the same plant community. Pollinator community composition can be different for herbivore-infested plants compared to uninfested plants (Steets et al. 2006, Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3), but not necessarily (Lucas-Barbosa et al. 2013, Burkle and Runyon 2016), and this may depend on the identity and feeding guild or feeding site of the herbivore (Rusman et al. 2018 - chapter 3). Herbivores also visit flowers frequently, and as larvae or adults can consume specific organs or the

complete flower (McCall and Irwin 2006). Florivores can feed exclusively on flowers, but also start feeding on leaves and move to the flowers later in development, or switch diet when flowers become available (Lucas-Barbosa et al. 2013). Florivore community assembly can be affected by early-season leaf herbivory (Stam et al. 2018). Interestingly, the florivore community of *Brassica oleracea* was affected differentially by the sequence of arrival of two leaf-feeding herbivores early in the season. Although here considered separately, it is important to realize that the pollinator and florivore communities are intertwined.

In addition to interactions with insects, flowers contain a rich microbial community consisting of fungi, bacteria, archaea, and viruses (Aleklett et al. 2014). These microbes can be antagonists by destroying flower tissues, opportunists that exploit the transient habitat without benefits or detriments to the flowers, or mutualists by competing for niche space with antagonistic microbes. The floral microbial community shows considerable variation in space, various flower organs, and time (Aleklett et al. 2014). Although there is no direct evidence that herbivore-induced changes in flower traits affect floral microbial community composition, this is most likely (Allard et al. 2018). Herbivore-induced changes in flower traits include many characteristics that affect the abundance and diversity of floral microbes such as nectar composition and volatile emission (Aleklett et al. 2014). Chemical properties of nectar are a prime determinant of microbial communities in nectaries. For example, the composition of secondary metabolites such as pyridine-type alkaloids affect bacterial community richness, diversity and composition in the nectar (Aizenberg-Gershtein et al. 2015), and herbivory can increase nectar alkaloid levels (Adler et al. 2006). Therefore, we expect that changes in flower traits in response to herbivory affect floral microbial communities. The extent of herbivore-induced changes in floral microbial community composition, differential responses of microbe species, and differential effects on microbes inhabiting varying floral organs deserve further investigation. Hence, herbivore-induced changes in flower traits have flower-community wide consequences.

Flower plasticity can link multiple interactions between flower visitors. Flowers are generally short-lived (Ashman 2004). Flower-associated organisms all interact with flowers during this short time window. Flower visitors that respond to herbivore-induced changes in flower traits, so-called receivers, can become inducers themselves when their activities induce additional changes in flower traits (Fig. 3). Such flower-visitor-induced changes in flower traits can subsequently affect other flower visitors (van Doorn 1997, Lucas-Barbosa et al. 2016, Lenaerts et al. 2017, Rering et al. 2018, Sobhy et al. 2018, Soper Gorden and Adler 2018), and even feedback to leaf herbivores (Soper Gorden and Adler 2016). Changes in flower phenotype in response to each interacting flower visitor will result in multiple linked indirect interaction units, where one interaction unit comprises an inducer, the mediator (the flower(s)), and a receiver (Fig. 3) (Utsumi et al.

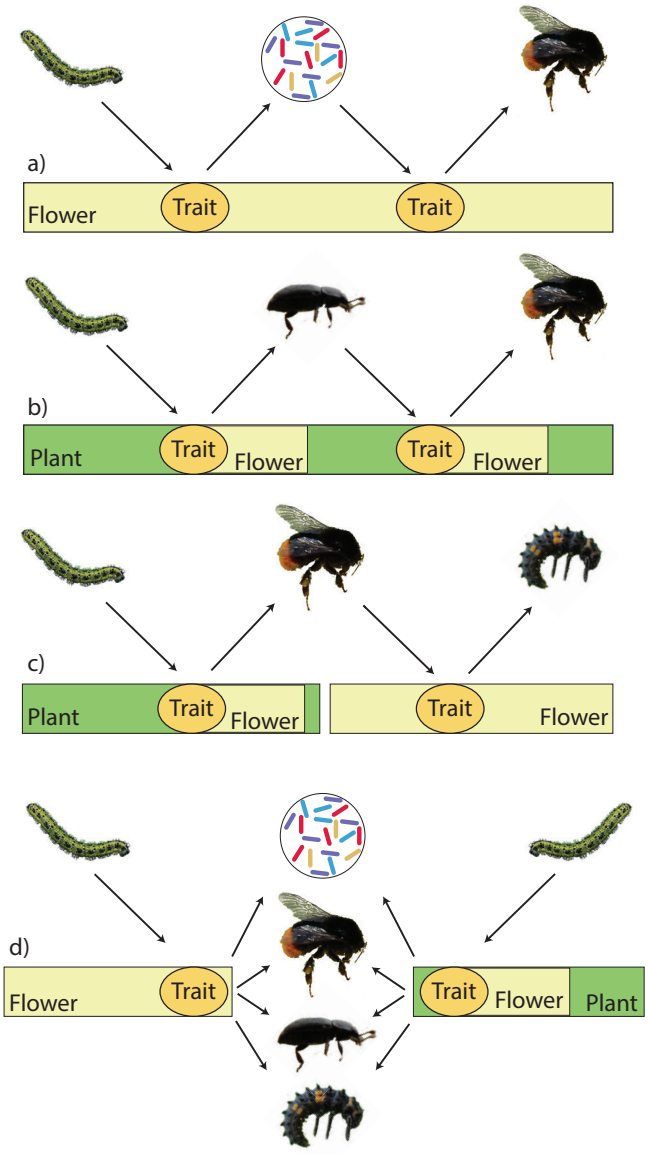

Fig. 3 Theoretical framework of how plant-mediated species interactions form a network of trait-mediated species interactions within flowers (a), the whole plant (b), or both (c, d). a) A flower-feeding herbivore that induces a local plant response (inducer) affects floral microbes (receiver), that in turn may affect the same or different flower traits that are received by a pollinator. b) A leaf-feeding herbivore that induces systemic plant responses (inducer) affects a flower-feeding herbivore (receiver), that in turn also induces systemic plant responses and affects the same or different flower traits that are perceived by a pollinator. c) A leaf-feeding herbivore that induces systemic plant responses (inducer) affects a pollinator (receiver), that in turn induces local plant responses and affect the same or different flower traits that are received by a predator. d) Local- or systemic-induced changes may affect multiple receivers at the same time and have flower-community wide consequences. Photograph credits: Erik Poelman and Quint Rusman.

2010). For example, herbivory can induce changes in nectar composition and volatile emission (Adler et al. 2006, Bruinsma et al. 2014), which subsequently affect nectar microbial community composition and pollinator visitation (Bruinsma et al. 2014, Aizenberg-Gershtein et al. 2015, Lucas-Barbosa et al. 2016, Rusman et al. 2019 - chapter 5). Different nectar microbial communities may induce further changes in nectar composition and volatiles, which affect parasitoid foraging and performance (Lenaerts et al. 2017, Sobhy et al. 2018), and pollinator visitation (Rering et al. 2018). Indeed, artificial florivory, pollination, and nectar robbery have been shown to affect multiple subsequent flower visitors at the same time, including pollinators, nectar thieves and **robbers**, florivores, and predators (Soper Gorden and Adler 2016, 2018). Interestingly, most effects were non-additive which suggests that the effect of one flower visitor can depend on the presence or behaviour of other flower visitors. Taken together, the extensive plasticity of flowers likely results in a highly connected web of interactions within the complete flower-associated community.

Although flower plasticity most likely has community-wide effects, broader patterns are difficult to predict. Leaf and root herbivory induce systemic changes in the plant, likely affecting all flowers in the inflorescences, with high potential to affect temporal or spatially displaced flower visitors (Chautá et al. 2017, Rusman et al. 2018 - chapter 3). For floral herbivory, herbivore-induced changes can be local, which would restrict the effects to visitors of that specific flower or a specific inflorescence. For example, changes in flower phenotype due to feeding damage by florivores can be restricted to individual flowers, and damaged flowers subsequently receive fewer pollinator visits (Cardel and Koptur 2010, Muola et al. 2017). Moreover, the tissue/organ that is damaged can influence how the flower appears to flower visitors (Oguro and Sakai 2009, Botto-Mahan et al. 2011). For example, nectar-guide removal in *Alstroemeria ligtu* reduced pollinator visitation, whereas lateral red tepal removal did not (Botto-Mahan et al. 2011). Interestingly, damaged flowers can also reduce visitation to undamaged flowers in the inflorescence (Sõber et al. 2010), either due to systemic changes in the whole inflorescence, or because pollinators judge potential food plants on the plant level and therefore avoid damaged plants as a whole. Pollinator- and microbe-induced changes in flower traits are expected to be mostly local. Some of these local effects, such as pollinator-induced changes in flower longevity, affect plant appearance as a whole, and thereby pollinator and florivore visitation to non-induced flowers (van Doorn 1997, Harder and Johnson 2005). Therefore, community-wide effects of flower plasticity likely vary considerably depending on local or systemic induction. Moreover, the effects of local induction might depend to a large extent on flower longevity and abundance. Plant species can differ greatly in flower longevity and abundance, from having just a few flowers that can last multiple months, to having hundreds of flowers that last for a few days or less (Ashman 2004). Community-wide

effects of local induction are probably much more extensive for plant species with a few long-lived flowers compared to plant species with a high floral turn-over. The extent of floral plasticity can also vary depending on flower longevity and abundance. Long-lived flowers are expected to be more plastic to continuously adapt to environmental variation (van Doorn 1997, Ashman 2004). Orchid flowers, for example, which are relatively long-lived, seem to respond readily to pollination by changes in flower longevity (Ashman 2004). Although potentially less affected by local pollinator-induced changes, short-lived flowers still readily change in response to systemic induction by herbivores (Barber et al. 2011). Local microbe-induced changes might affect short- and long-lived flowers to the same extent, because microbes directly modulate flower traits rather than indirectly *via* changes in plant physiology. The consequences of such microbe-induced changes will be more apparent for plants with long-lived flowers, however, due to the lower rate of flower turn-over and smaller numbers of flowers (Ashman 2004). Thus, flower longevity and abundance can restrict and dilute community-wide effects of local-induced changes in flower traits, whereas systemic-induced changes readily affect temporally or spatially displaced flower visitors.

Concluding remarks and future perspectives

To understand the ecology of flower plasticity, we need to adopt a community context approach. The evolution of flowers cannot be fully explained by focussing solely on pollinators, but rather by combined selection of the flower-associated community, e.g. pollinators, florivores, and microbes (Strauss et al. 2005, Strauss and Whittall 2006, Soper Gorden and Adler 2018). Moreover, communities are characterized by ample indirect interactions among their members. These indirect interactions are as important as direct interactions are in shaping evolution (Siepielski and Benkman 2004, Poelman and Kessler 2016, Guimarães Jr et al. 2017). Hence, organisms such as herbivores not directly associated with the flowers can contribute to flower evolution by indirectly affecting interactions with flower visitors (**diffuse selection**). The herbivore-species-specific nature of flower plasticity in response to herbivory suggests a high number of potential selective agents, but also large temporal and spatial fluctuations in selection pressure exhibited by such indirect interactions. Particular plant traits such as flower longevity and abundance, or the ability to separate foliar from floral plant responses, or defence and reproduction for the plant as a whole, might determine the extent of diffuse selection by indirect interactions. Separation of foliar from floral plant responses, and defence and reproduction, can partly be achieved by tissue-specific gene expression and regulatory components, and plant ontogenetic trajectories in growth-defence-reproduction strategies. Still, it might be difficult for plants to completely separate processes in leaves and flowers, and defence and reproduction, due to various physiological links that allow

plants to function as one integrated entity. Exploring the ecology of plastic flowers will extend our understanding of the evolution of plant defence and reproduction.

Outstanding questions

- What are the molecular mechanisms underlying defence regulation in flower tissues?
- How tight is the link between physiological regulation of defence and reproduction, which mechanisms are especially important, and does this vary between systems or under different environmental conditions?
- Can we identify patterns of specificity of induction by flower visitors other than insect herbivores such as pollinators and microbes, and what are differences and similarities compared to specificity of induction by herbivores?
- To what extent do herbivore-induced changes in flower traits affect floral microbial community composition, and can we identify differential responses of microbe species and different floral organs?
- Can we identify broader patterns of herbivore-induced changes in flower traits on flower-community assembly, are these predictable, and are certain groups of flower-associated organisms more affected than others?
- Can herbivore-induced changes in flower traits drive eco-evolutionary dynamics?

To better understand flower plasticity in response to herbivory, we need to deepen our knowledge on specificity of induction, for example by using combinations of herbivores that show patterns of induction of changes in foliar and/or root traits related to feeding guild, feeding site, or host plant specialization. Such studies should measure a multitude of flower traits because specificity of induction can only be judged when considering the complete flower phenotype. We have just started to unravel the molecular mechanisms underlying specificity in flower plasticity, especially how phytohormones, secondary metabolites, and defence genes are expressed in the flowers (Oh et al. 2013, Ederli et al. 2015, Zhai et al. 2015, Bruckhoff et al. 2016, Li et al. 2017). More insight into such tissue-specific plant responses will shed light on which of the potential links between defence and reproduction (Box 1) are common and important in flower plasticity in response to herbivory. Moreover, research on specificity of induction will provide broader insights in how flowering plants deal with ecological variation, and optimize the attraction of mutualists while dealing with antagonists. The consequences of floral plasticity should be investigated in a community context, and should consider adaptive plasticity, with consequences for plant reproduction. An interesting approach would be to focus on keystone herbivores: herbivores that have a large effect on the interaction network with associated fitness consequences for the plant (Poelman and Kessler 2016). Keystone herbivores that especially affect the flower-visitor community can be compared with keystone herbivores that have large effects on foliar and/or root communities, and non-keystone herbivores to identify how such herbivores drive selection in complex communities, and what the contribution of flower plasticity and the flower-associated community is. Another interesting approach will be to compare responses of flower-associated communities varying in their overall

degree of host plant specialization (Lucas-Barbosa 2016), because these might differentially impact plant fitness, and open up different evolutionary trajectories. In addition to systemic induction, the role of local flower plasticity in response to herbivory, but also pollination and microbial induction, need further attention (See Outstanding Questions). The community-wide effects of local compared to systemic flower plasticity have so far not been explored, and it will be interesting to compare flower plasticity of plants with particular traits, such as flower longevity and abundance, in this perspective.

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Chapter 3

Dealing with mutualists and antagonists: Specificity of plant-mediated interactions between herbivores and flower visitors, and consequences for plant fitness

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Abstract

Plants need to deal with antagonists, such as herbivores, while maintaining interactions with mutualists, such as pollinators that help plants to maximize their reproductive output. Although many plant species have inducible defences to save metabolic costs of defence in the absence of herbivores, plant responses induced by herbivore attack can have ecological costs. For example, herbivore-induced responses can affect flower traits and alter interactions with flower visitors. Such plant-mediated interactions between herbivores and flower visitors can affect plant reproductive output. Current knowledge on the generality and specificity of plant-mediated herbivore - flower-visitor interactions and its consequences for plant fitness is limited. In this study, we investigated whether a broad range of herbivores feeding on the annual plant *Brassica nigra* affect interactions with flower visitors, whether the direction of interactions is predicted by the feeding modes (chewing and sap-feeding) and sites (above- and belowground) of the herbivores, and whether it results in fitness consequences for the plant. Our results show that attack of *B. nigra* by a range of different herbivores influenced plant interactions with mutualist pollinators and an antagonist florivore, the pollen beetle *Meligethes aeneus*. Pollinator community composition was affected by herbivory, whereas overall pollinator attraction was maintained. Pollinator community composition of uninfested plants differed from that of chewing and root herbivore-infested plants. Main responders in the pollinator communities to changes induced by herbivory were syrphid flies, bumblebees, and solitary bees. Although the preference of pollen beetle adults was not affected by herbivory, beetle larvae performed best on plants infested with the nematode *Heterodera schachtii*. The changes in pollinator community composition and syrphid fly visitation can explain the observed increase in seed set of root herbivore-infested plants. Interactions of flowering *B. nigra* plants with mutualist and antagonist insects are well integrated and conflicting interactions do not reduce reproductive output. Our results suggest some degree of specificity in herbivore - flower-visitor interactions with consequences for plant fitness. Specificity of plant responses were determined at the species level as well as the herbivore functional group level, and differed depending on the flower visitor. Because plant reproduction was affected by indirect plant-mediated interactions, these can potentially result in selection on plant strategies to optimize growth, defence, and reproduction.

Keywords: flower visitors, flower traits, herbivore-induced plant responses, plant defence, phenotypic plasticity, specificity

Introduction

To maximize reproductive output, plants need to maintain interactions with mutualists, such as pollinators, and at the same time deal with antagonists, such as herbivores (Herrera et al. 2002, Strauss et al. 2004, Sauve et al. 2016). On the one hand, traits evolved in plants to attract mutualists, can be exploited by antagonists to find host plants (Theis and Adler 2012, Nunes et al. 2016). On the other hand, defensive traits of plants to deal with antagonists, may deter mutualists (Price et al. 1980, Kessler and Halitschke 2009). Conflicts in interactions between plants and their antagonists and mutualists are particularly apparent when antagonists and mutualists interact with the same plant organ, such as in the case of flowers. For example, floral scent bouquets are attractive to mutualistic flower visitors, and can be attractive or repellent to antagonistic flower visitors (Junker and Blüthgen 2010, Theis and Adler 2012, Kessler et al. 2013, Pichersky and Raguso 2016). Phenolic compounds that colour flowers and evolved as cues to pollinators may serve as defensive chemical compounds against flower-feeding herbivores (Lattanzio et al. 2008, McCall et al. 2013), but also be exploited by flower-feeding herbivores (Yaku et al. 2007, Doering et al. 2012). Changes in flower traits which are exploited in multiple interactions, may lead to conflict for the plant while optimizing defence and reproduction. Failure to attract sufficient pollinators may negatively impact plant reproduction (Collison and Martin 1979, Ohara and Higashi 1994, Wilcock and Neiland 2002). Reduced attraction of florivores and seed predators may be beneficial for the plant, as florivores and seed predators consume expensive reproductive organs of the plant (Mothershead and Marquis 2000, Cariveau et al. 2004). Thus, conflicts in interactions of plants with antagonists and mutualists might have consequences for plant fitness.

Conflicts of dealing with insect herbivory and maintaining pollination may also arise when herbivores feed on different organs than the flowers, such as leaves or roots. In most plant species, defensive traits are induced upon attack to save metabolic costs of defences in the absence of attackers, and these herbivore-induced defences allow plants to tailor their defences to specific attackers. However, plant responses to herbivory have ecological costs and can alter interactions with other species (Heil 2002, Strauss et al. 2002). Because herbivore-induced plant responses are often systemic, they may involve induced changes in flower traits (Agrawal et al. 1999, Adler et al. 2006, Kessler and Halitschke 2009, Lucas-Barbosa et al. 2016), and can affect the behaviour of both mutualistic and antagonistic flower visitors (Kessler and Halitschke 2009). Several studies have identified flower traits that are affected by herbivory (Pareja et al. 2012, Bruinsma et al. 2014, Hoffmeister et al. 2016, Lucas-Barbosa et al. 2016), and some flower traits may change in response to herbivory because they share biosynthetic pathways or resources with defensive traits (Herms and Mattson 1992, Fineblum and Rausher 1997, Johnson et al. 2015). Subsequently, herbivore-induced plants have been found to negatively affect

pollinator attraction and visitation (Barber et al. 2012, Liao et al. 2013, Schiestl et al. 2014), but there are also reports of neutral (Ivey and Carr 2005, Lucas-Barbosa et al. 2016) or positive effects on pollinator attraction (Poveda et al. 2003, Barber et al. 2011). Only few studies addressed the effect of plant responses to herbivory on antagonist flower visitors, such as florivores and seed predators. Effects of plant responses to herbivory on the colonization and performance of floral antagonists were either negative (McArt et al. 2013, Boyer et al. 2016) or positive (Gorden 2013) in these recent studies. To date, it is, however, not evident what determines the specificity of plant-mediated interactions between herbivores and flower visitors (Lucas-Barbosa 2016).

Specificity of plant responses induced by herbivory is hypothesized to underlie patterns in plant-mediated herbivore - flower-visitor interactions. To fine-tune defences against the diversity of herbivores, we expect that plants can recognize and respond to each attacker specifically (Agrawal 2000, Travers-Martin and Müller 2007, Uesugi et al. 2013) and plant responses can vary based on pattern of damage (Mithöfer et al. 2005) and compounds released by herbivores during feeding (Howe and Jander 2008, Wu and Baldwin 2010). Herbivores with similar feeding modes (Howe and Jander 2008, Bidart-Bouzat and Kliebenstein 2011, Erb et al. 2012, McCormick et al. 2012) or that feed on the same plant organs (Farré-Armengol et al. 2015, Johnson et al. 2016, Lucas-Barbosa et al. 2016) induce more similar plant responses than herbivores that differ in feeding guild or feeding site. For example, chewing insects primarily induce the jasmonic-acid (JA) dependent defence pathway, whereas sap-feeding insects may suppress JA and/ or induce the salicylic-acid (SA) dependent defence pathway (Turlings et al. 1998, Rodriguez-Saona et al. 2003, Heidel and Baldwin 2004, De Vos et al. 2005, Rowen and Kaplan 2016). Indeed, feeding guild was found to be a good predictor for differential plant responses, including differences in JA- and SA- related genes and downstream secondary plant metabolites (Bidart-Bouzat and Kliebenstein 2011, Ali and Agrawal 2012, Rowen and Kaplan 2016). Plant responses induced by organisms feeding belowground are regulated through different phytohormonal networks compared to responses induced by organisms feeding aboveground (Johnson et al. 2016). It remains to be identified if feeding guild and feeding site chosen by herbivores predicts the direction of effects on mutualist and antagonist flower visitors, and how these responses translate in effects on plant seed production.

In this study, we tested whether a broad range of herbivores feeding on the annual *Brassica nigra* affect interactions with flower visitors, whether the direction of interactions is predicted by the feeding modes (chewing and sap-feeding) and sites (above- and belowground) of the herbivores, and whether herbivore - flower-visitor interactions result in fitness consequences for the plant. The herbivores were divided into three groups that we here term "herbivore functional groups" (HFGs): chewing herbivores, sap-feeding herbivores and root herbivores. We specifically studied in what direction these herbivores

affect (i) attraction and visitation rates of mutualists (pollinators), (ii) preference and performance of an antagonist, the florivore *Meligethes aeneus*, and (iii) seed set. We discuss the role of plant-mediated interactions between herbivores and flower visitors in plant strategies to optimize growth, defence and reproduction.

Materials and Methods

Plant and insects

In all experiments, we used seeds of Black mustard (*Brassica nigra*) accession CGN06619 that originates from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands) and were propagated by open field pollination. Seeds were germinated in trays. One-week old plants were transplanted to and cultivated in pots (∅ 17 cm – 2 L) filled with potting soil (Lentse potgrond) and sand in a 1:1 volume ratio under greenhouse conditions (23 ± 2 °C, 50 -70 % r.h., L16:D8). Two-week-old plants were moved to an outside area protected by insect screen. Plants that had 1-10 open flowers (± 6 weeks old) were transplanted to the field and immediately infested with herbivores. We exposed plants to 10 herbivore species divided over three HFGs; chewing herbivores (*Athalia rosae*, *Mamestra brassicae*, *Pieris brassicae* or *Plutella xylostella*), sap-feeding herbivores (*Brevicoryne brassicae*, *Myzus persicae*, or *Lipaphis erysimi*), and root herbivores (*Delia radicum*, *Heterodera schachtii*, or *Pratylenchus penetrans*). The caterpillars *P. brassicae*, *M. brassicae*, and *P. xylostella*, and aphids *B. brassicae, M. persicae* and *L. erysimi*, originated from the surroundings of Wageningen (The Netherlands), and they are routinely reared in the laboratory of Entomology (Wageningen University) under greenhouse conditions (22 ± 1 °C, 50 -70 % r.h., L16:D8). Caterpillars and *B. brassicae* were reared on Brussels Sprouts plants (*Brassica oleracea* variety *gemmifera* cultivar Cyrus); *M. persicae* and *L. erysimi* were reared on *Raphanus sativus*. The sawfly *A. rosae* originated from surroundings of Würzburg (Bavaria, Germany). The larvae were reared on *R. sativus* under greenhouse conditions (22 ± 1 °C, 50 -70 % r.h., L16:D8). The cabbage root fly *D. radicum* originated from St. Méloir des Ondes (Brittany, France). Larvae were reared on turnips (*Brassica rapa*) or rutabaga (*Brassica napus*) in a climate cabinet (22 ± 1 °C, 50 -70 % r.h., L16:D8). Cysts of the nematode *H. schachtii* originated from the rearing of the Institute for Rational Sugar production (IRS) (Bergen Op Zoom, The Netherlands). The population used was IRS 07-01-04.02 and originates from Woensdrecht, The Netherlands. Nematodes were reared on rapeseed (*B. napus* cultivar Jennifer). Cysts were hatched using a 3 mM $ZnCl_2$solution. After hatching, nematodes were flushed out of the hatching sieves using tap water, and solutions containing about 1000 nematodes (J2 stage) in 4 mL of water were used to infest plants. The nematode *P. penetrans* originated from the stock rearing of Wageningen Plant Research (Lelystad, The Netherlands). Plants were infested with a solution containing about 1000 nematodes - comprising individuals of all growth

stages - in 4 mL of water. Adult pollen beetles (*Meligethes aeneus*) used to infest plants were collected from flowering *B. nigra* plants surrounding the field site (Wageningen University, Wageningen, The Netherlands).

Common garden experiments - Field designs

A common garden experiment was designed to test the effects of herbivore infestation on the visitation/abundance of mutualist and antagonist flower visitors. We planted 176 plots of *B. nigra* in a bare field. Plots of five plants were composed of one central plant and four surrounding plants, at a distance of 20 cm. Distance between central plants of neighbouring plots was 1.5 meter. The plots were distributed over 2 experimental blocks defined by the week of planting. Each block was composed of 88 plots that were organized in 11 rows and 9 columns. To reduce spatial effects, the plots of columns 1-4 were planted between day 1 and 4, column 5 was kept empty, and plants of columns 6-9 were planted between day 4 and 7. Each day 13 plots were planted, except for day 4, when we planted 10 plots. Plants of the second block were planted 14 days later following the same protocol. Blocks were separated by 3 meters of bare soil. On the day that plants were transplanted into the field, each plot of five plants was infested with one of the 10 herbivore species, or kept as an uninfested (control) plot. Herbivore-infested and control plots were randomly divided over each block using a Latin square design, and equally divided over the planting dates. Each herbivore-infested and uninfested control plot was replicated 16 times.

A second common garden experiment was designed to test the effect of herbivore infestation on the performance of pollen beetle larvae. The layout of this experiment was similar to that used to monitor pollinator visitation and abundance of pollen beetles. This field was composed of 110 plots of five plants each. All plots of plants were transplanted to the field within three days and assigned to one of the 10 herbivore species or kept as an uninfested (control) plot. Herbivore-infested and control plots were randomly divided over each block using a Latin square design, and equally divided over the planting dates. Each herbivore species and uninfested control was replicated 10 times. After infesting the plots, we joined the top 3 to 4 inflorescences of the central plant of each plot together in a mesh bag to prevent natural pollen beetle infestation. Mesh bags had one opening, through which the stems of the inflorescences entered the bag, which was closed using a rope. To support the mesh bag, the top of a bamboo stick of approximately 1 meter long was also inserted in the bag together with the inflorescences, and put next to the plant in the soil.

Plant treatments

To infest *B. nigra* plants with aboveground herbivores, we placed 10 first instar chewing herbivores or 10 adult sap-feeding herbivores on two small leaves (five per leaf); at half of the plant height in between true leaves and the inflorescence. To infest plants with *D. radicum*, we placed 10 first instar larvae at the base of the stem. To infest plants with nematodes, 4 mL of solution containing 1000 nematodes was added in four holes (1 mL per hole) around the main stem. Holes, approximately 10 cm deep with a diameter of 0.5 cm, were made at 2 cm from the stem of each plant. We recorded survival of aboveground herbivores, per plant of a given plot, six days after infestation. When no herbivores were recorded, plants were re-infested with five second instar chewing larvae or five adult sap-feeding herbivores. Because we needed to re-infest a large number of plants in block 1, we decided to put a mesh tent (95 x 95 x 190 cm) for 24 hours over each plot of block 2, right after planting, to provide time for the herbivores to settle on the plants. For all belowground treatments, root samples were taken from several plants at the end of the experiment, and assessed for damage by *D. radicum* and nematode presence.

Effect of herbivore infestation on floral mutualists

To investigate if pollinator visitation to flowers of *B. nigra* was influenced by plant infestation with herbivores, we recorded pollinator behaviour on plots of plants infested with different herbivores and to non-infested plots at two time points: 7 and 14 days after infestation of the plots. Each plot was monitored for 10 minutes. When a pollinator entered the plot and had contact with a flower, identity of the pollinator, number of flowers visited, and time spent per flower were recorded. If during the observation of a pollinator other pollinators entered the plot, their visitation and identity was recorded. If the same pollinator individual returned to the plot under observation after having visited a different plot, we scored that visit as a new visit. Pollinator identity was divided over 6 groups: honeybees (*Apis mellifera*), bumblebees (*Bombus terrestris, Bombus lapidarius, Bombus pratorum*, *Bombus hortorum*, and other *Bombus* spp.), syrphid flies (several *Eristalis* spp. and several other Syrphidae spp.), solitary bees (several *Andrena* and *Lasioglossum* spp. but also other *Apidae* excluding *Bombus* spp.), other flies (other Diptera than Syrphidae), and butterflies (*Pieris* spp. and other Lepidoptera). Observations were performed using a handheld computer (Psion Workabout Pro^{tm} 3, London, UK) programmed with The Observer XT software (version 10, Noldus Information Technology, Wageningen, The Netherlands). Observations were carried out between 9 am and 1 pm, or 2 pm and 5 pm, when weather conditions were suitable for pollinator activity (15 -30 ^{o}C – wind speed $\leq$ 6 m$\cdot$s^{-1}).

Effect of herbivore infestation on a floral antagonist

To investigate if pollen beetle (*Meligethes aeneus*) colonization was influenced by plant infestation with herbivores, we monitored pollen beetle abundance on plots of plants infested with different herbivores and on non-infested plots at two time points: 7 and 14 days after infestation of the plots. The abundance of pollen beetles on infested and non-infested plots was recorded in the same field where we observed pollinator behaviour. Pollen beetle abundance was monitored by counting the number of adult pollen beetles on all plants of a given plot. Observations were carried out between 2 pm and 6 pm when weather conditions were suitable for pollen beetle activity (15 -30 ^{o}C – wind speed $\leq$ 6 $m \cdot s^{-1}$).

To investigate if pollen beetle oviposition and larval performance was influenced by plant infestation with herbivores, we added 6 - 8 pollen beetle adults to the mesh bags with inflorescences, assuming a sex-ratio of 1:1, 7 days after infestation of the plots. Plots of day 1 received six beetles (three pairs). At day 2 however, we were able to collect enough beetles, but not enough pairs (we were unable to sex pollen beetles without dissecting them, except when mating couples were observed). Thus, we decided to take eight random beetles instead of three pairs of mating beetles for day 2, 3, and 4. The two sampling methods yielded differences in number of offspring found (Table A4), and we accounted for this difference by including day as random factor in the statistical analysis. Due to shortage of beetles on day 1, we added beetles on 11 plants (one replicate of each herbivore species and uninfested control) 9 days after infestation of the plots, instead of 7 days, and we accounted for this difference in the statistical analysis by labelling these samples as day 4. Adult beetles were allowed to deposit eggs in flower buds for 72 h, and were then removed. Inflorescences were harvested 7 days after removing the adult beetles by cutting the stems of the inflorescences, at the point where bags were closed, and stored at -20 ^{o}C . Later, samples were defrosted, and pollen beetle larvae counted and weighed individually.

Effect of herbivore infestation on plant seed set

To investigate if life-time seed production was influenced by plant infestation with herbivores, we assessed seed production for plants used for pollinator behaviour observations and pollen beetle abundance monitoring. We harvested seeds of three plants of each plot; the central plant and two side plants (randomly selected and not adjacent to each other). First harvesting date was selected before the first siliques would lose their seeds; *i.e.* 47 days after planting in the field. At this time point, we harvested all ripe siliques. Immature siliques and flowers were left on the plants and harvested up to 1 month later, whenever they were ripe. We stored the seeds in paper bags in a warm

and dry storage room. After drying, we calculated total number of seeds per plant by weighing 100 seeds, and the total weight of seeds harvested per plant. We estimated the total number of seeds by dividing total seed weight by the weight of 100 seeds. Some plants of the second block lost seeds before harvest. We estimated the number of seeds lost by counting open siliques. In addition, we collected 10 siliques of one random plant of 15 random plots, counted the number of seeds per silique and calculated the average number of seeds per silique. By multiplying the number of lost siliques and average number of seeds per silique, we calculated the number of seeds lost.

Statistical analysis

We analysed number of flower visitors and total flowers visited for all pollinators combined, and when regarding specific responses by pollinator groups, we analysed number of visitors, flowers visited in total and per visit, and time spent per visit and flower, number of pollen beetle adults and larvae, and weight of pollen beetle larvae using (generalized) linear (mixed) models ((G)L(M)Ms). We analysed pollinator community composition using chi-square tests. All analyses were carried out in R (version 3.1.3 x64, 2015, The R Foundation for Statistical Computing Platform).

For count data such as the number of insects, flowers and seeds, we used a Poisson distribution with a log link function or negative binomial distribution with a log link function to correct for over-dispersion, when needed. Herbivore species, HFG, and time-point (except for pollen beetle larvae and seed data) as well as their interactions were included in the model as fixed factors. Herbivore species was included in the model as a nested factor within HFG. Interactions were removed from the model if they were statistically non-significant ($P > 0.05$). For *post hoc* analyses of individual fixed factors we used Tukey's *post hoc* tests, for interactions between fixed factors we used least squares means. Random factors were selected using a backward approach; all random factors such as day, time (morning vs afternoon) (only for pollinators), block (1 or 2), plot (for number of pollen beetle adults and larvae), day*treatment or day*HFG, were initially added to the model and removed if they explained less than 10^{-3} of the variation or were statistically non-significant ($P > 0.05$). For the seed set data only block was used as random factor. We used the lme4 (Bates et al. 2015), lmtest (Zeileis and Hothorn 2002), multcomp (Hothorn et al. 2008), and lsmeans (Lenth 2016) packages for these analysis. For continuous data such as time spent per visit and per flower by pollinators, and weight of pollen beetle larvae, we used a Gaussian distribution with identity link function or a Gamma distribution with a log link function if the data did not follow a normal distribution. The same fixed and random factors, and packages as for count data were used. Due to the low number of observations for syrphid fly visitation of plants that had been flowering for 7 days, we analysed total number of flowers visited,

number of flowers visited per visit, time spent per plant or flower, only for syrphid fly visits to plants that had been flowering for 14 days. The number of flower visitors for each pollinator group was used to reconstruct pollinator community compositions for infested and uninfested plots. We analysed pollinator community composition by comparing the pollinator community composition of infested and uninfested plots with a chi-square test. Expected pollinator community composition was calculated by summing pollinators within each group for all plots, and dividing this number by the total number of pollinators. This results in an expected percentage for each pollinator group. This percentage was then multiplied by the total number of pollinators recorded for infested or uninfested plots. We did not take the pollinator community composition of uninfested plots as expected pollinator community composition because the pollinator community distributes over the different treatments including the uninfested plots based on pollinator preference in the choice situation, e.g. the community composition of the uninfested plots is affected by the presence of the infested plots. If pollinator community composition was explained by herbivore or HFG treatment, pair-wise comparisons among all herbivore or HFG treatments were performed using chi-square tests. To correct for multiple tests of pair-wise comparisons, we adjusted the *P*-values using the false discovery rate (FDR) correction. We used the fifer package for these analyses (Fife 2014). In addition, to assess which pollinator groups contributed to differences between herbivore or HFG treatments, we calculated the adjusted residuals for each pollinator group in each treatment, and used a threshold value of ± 2, as described by Sharpe (2015). Pollinator groups which composed less than 2 % of the community were excluded from the analysis, e.g. other flies (1.4 %) and butterflies (0.1 %).

Results

Effect of herbivore infestation on floral mutualists - pollinator community composition

The four most abundant pollinator groups were honeybees (70 %), syrphid flies (19 %), bumblebees (7 %), and solitary bees (3 %). Pollinator community composition of *B. nigra* varied depending on whether plants had been exposed to root, sap-feeding or chewing herbivores (Fig. 1, chi-square test, $P = 0.007$), but not depending on whether plants had been exposed to specific herbivore species (Fig. 1, chi-square test, $P = 0.148$). The composition of the pollinator community visiting flowers of uninfested plants differed from the community visiting flowers of plants infested with chewing (Fig. 1, chi-square test, $P = 0.029$) and root herbivores (Fig. 1, chi-square test, $P = 0.019$). The composition of the pollinator community visiting flowers of plants infested with chewing herbivores was similar to that of plants infested with root herbivores (Fig. 1, chi-square test, $P = 0.236$). The composition of the pollinator community visiting flowers of plants infested with sap-feeding herbivores were visited by similar communities as flowers of uninfested

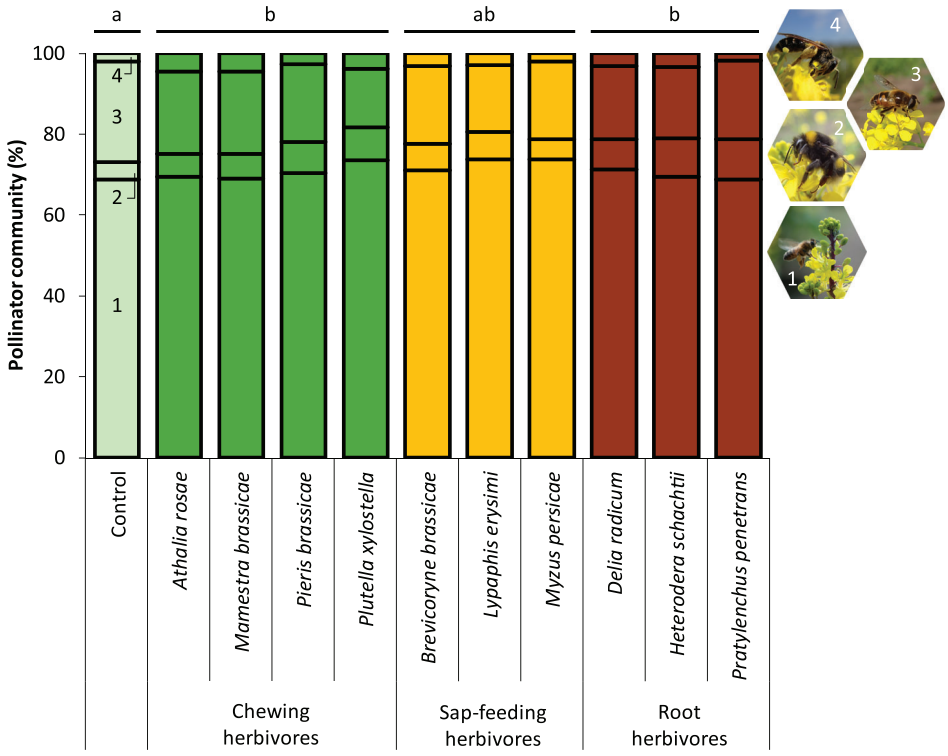

Fig. 1 Composition of pollinator communities of uninfested plots (control) of *Brassica nigra* and plots infested by different herbivores that belong to one of three different herbivore functional groups (HFGs): chewing, sap-feeding or root herbivory. Communities consist of honeybees (1), bumblebees (2), syrphid flies (3) and solitary bees (4). Letters above bars indicate significant differences at $P \leq 0.05$ based on chi-square tests.

plants (Fig. 1, chi-square test, $P = 0.091$), and flowers of plants infested with chewing (Fig. 1, chi-square test, $P = 0.335$) and root herbivores (Fig. 1, chi-square test, $P = 0.177$). Pollinators contributing most to the observed effects were bumblebees, syrphid flies and solitary bees, and different pollinators could be associated with different HFGs (Fig. 1, Appendix A Table A1). For instance, bumblebees contributed less strongly and syrphid flies more strongly to the composition of the pollinator community of uninfested plants, whereas bumblebees contributed more strongly to the composition of the pollinator community of root herbivore-infested plants. Solitary bees contributed more strongly to the composition of the pollinator community of chewing-herbivore infested plants.

Effect of herbivore infestation on floral mutualists - pollinator attraction

To investigate whether plant exposure to different herbivore species influences pollinator visitation before contact with the plant, we observed 3,646 pollinators visiting flowers of

the experimental plants. On average, about 16 pollinators visited flowers in plots during the 10 minutes observation time. The total number of pollinators visiting flowers of plants infested with different herbivores was overall similar (Fig. 2, Table A2 and A3). When regarding specific responses by pollinator groups, similar numbers of honeybees and bumblebees visited flowers of plants infested with different herbivores (Fig. A1, Table A2 and A3). However, herbivore infestation did affect the number of syrphid fly visitors (Fig. A1, Table A2 and A3). Overall, fewer syrphid flies visited flowers of plants infested with chewing herbivores (Fig. A1, Tukey's *post hoc* test, $P = 0.004$), or sap-feeding herbivore (Fig. A1, Tukey's *post hoc* test, $P = 0.034$) when compared with uninfested plants. Moreover, fewer syrphid flies tended to visit flowers of plants infested with any of the root herbivore species when compared with uninfested plants (Fig. A1,

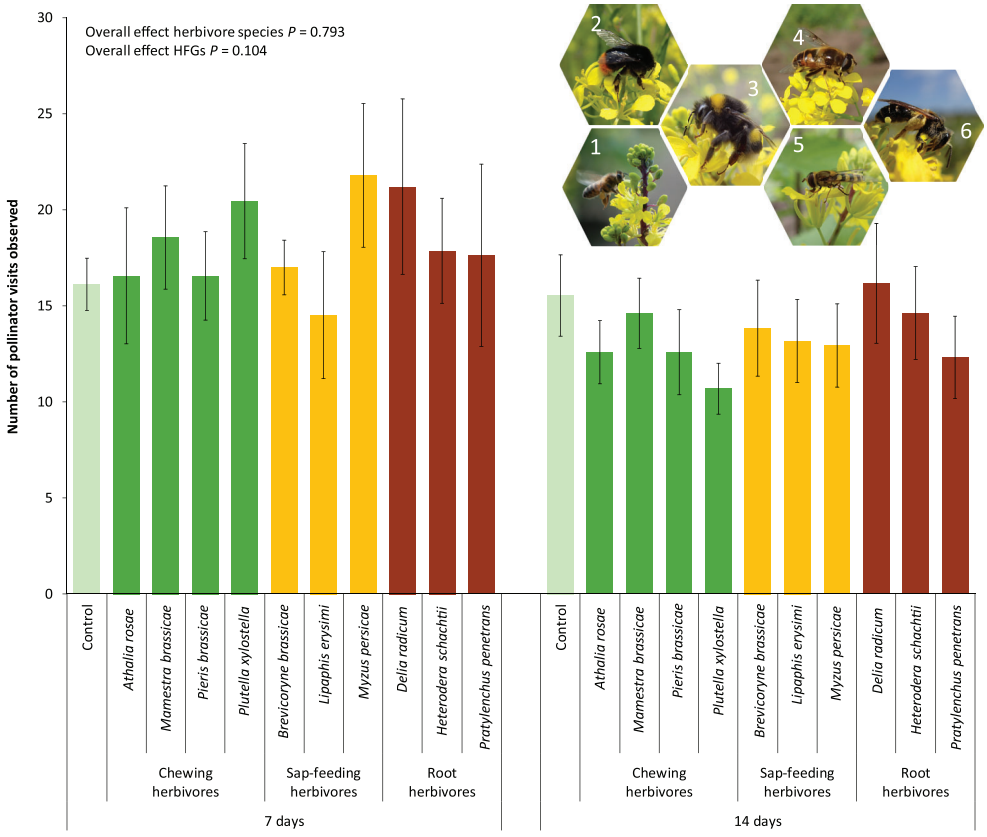

Fig. 2 Number of pollinator visits observed (mean ± SE) on flowers of uninfested plots (control) of *Brassica nigra* and on flowers of plots of plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs): chewing, sap-feeding or root herbivory. Most common pollinators recorded were 1) *Apis mellifera*, 2) *Bombus lapidarius*, 3) *B. terrestris*, 4) *Eristalis* spp., 5) other Syrphidae, 6) various solitary bees. Observations lasted for 10 minutes and were made at two time points: 7 and 14 days after infestation. Number of replicates per herbivore treatment varied between 9 and 13.

Tukey's *post hoc* test, $P = 0.068$). Fewer syrphid flies visited plants infested with *P. xylostella* compared with visitation to uninfested control plants (Fig. A1, Tukey's *post hoc* test, $P = 0.003$). When time point was included in this analysis, we also detected effects of plant responses to herbivores on the visitation of syrphid flies. Fewer syrphid flies visited flowers of plants infested with chewing herbivores (Fig. A1, Tukey's *post hoc* test, $P = 0.013$), or sap-feeding herbivores (Fig. A1, Tukey's *post hoc* test, $P = 0.036$) compared with uninfested plants, but only when plants had been flowering for 14 days. Fewer syrphid flies visited plants infested with *P. xylostella* compared with uninfested control plants, but only when plants had been flowering for 14 days (Fig. A1, Tukey's *post hoc* test, $P = 0.003$). Number of pollinators visiting flowers in plots of herbivore-infested and uninfested plants also varied with time. Overall more pollinators visited flowers of *B. nigra* plants that had been flowering for 7 days than of plants that had been flowering for 14 days (Fig. 2, Table A2 and A3, Tukey's *post hoc* test, $P = 0.014$), irrespectively of whether the plants had or had not been exposed to herbivores. We recorded more honeybees visiting flowers of plants that had been flowering for 7 days than of plants that had been flowering for 14 days (Fig. A1, Table A2 and A3, Tukey's *post hoc* test, $P = 0.005$). For syrphid flies we observed the opposite pattern: fewer syrphid flies visited flowers of plants that had been flowering for 7 days than of plants that had been flowering for 14 days (Fig. A1, Table A2 and A3, Tukey's *post hoc* test, $P > 0.001$). The number of bumblebees visiting *B. nigra* flowers remained constant over time (Fig. A1, Table A2 and A3).

Effect of herbivore infestation on floral mutualists - visitation times and flower visits

To investigate whether plant exposure to different herbivores influences pollinator visitation during contact with the plant, we observed 1,310 visits to 11,642 flowers. On average, about 50 flowers were visited in the 10-minute- observation time. The number of flowers visited for plants infested with different herbivores was overall similar (Fig. A2, Table A2 and A3). Regarding specific responses by pollinator groups, we observed that honeybees and syrphid flies visited similar numbers of flowers in total, as well as per visit, of plants infested with different herbivores (Fig. A2, A3 and A4, Table A2 and A3). Honeybees spent similar amounts of time per plot, and flower visit, of plants infested with different herbivores (Fig. A3, Table A2 and A3). Syrphid flies spent different amounts of time per plot, but not per flower visit, on plants infested with different herbivores when plants had been flowering for 14 days (Fig. A4, Table A2 and A3). For instance, syrphid flies spent more time visiting flowers of plants infested with the root herbivore *D. radicum* compared with plots of plants infested with the aphids *B. brassicae* (Tukey's *post hoc* test, $P < 0.010$) or *L. erysimi* (Tukey's *post hoc* test, $P = 0.044$). Overall, syrphid flies tended to spend more time visiting flowers of plants infested with root herbivores compared with

flowers of plants infested with sap-feeding herbivores (Tukey's *post hoc* test, $P = 0.055$).

Number of flowers visited and visitation times in plots of herbivore-infested and uninfested plants also varied with time. The number of flowers visited by honeybees was higher when plants had been flowering for 7 days than when plants had been flowering for 14 days, irrespectively of whether the plants had or had not been exposed to herbivores (Fig. A2, Table A2 and A3, Tukey's *post hoc* test, $P = 0.007$). Honeybees spent more time visiting flowers of plants infested with the caterpillar *P. xylostella* when plants had been flowering for 7 days compared with when plants had been flowering for 14 days (Fig. A2, Table A2 and A3, Tukey's *post hoc* test, $P = 0.002$ and $P = 0.047$ respectively) and tended to spend more time visiting flowers of uninfested plants when plants had been flowering for 7 days compared with plants that had been flowering for 14 days (Fig. A2, Table A2 and A3, Tukey's *post hoc* test, $P = 0.057$ and $P = 0.072$ respectively).

Effect of herbivore infestation on a floral antagonist

To investigate whether plant exposure to different herbivores influences the preference of

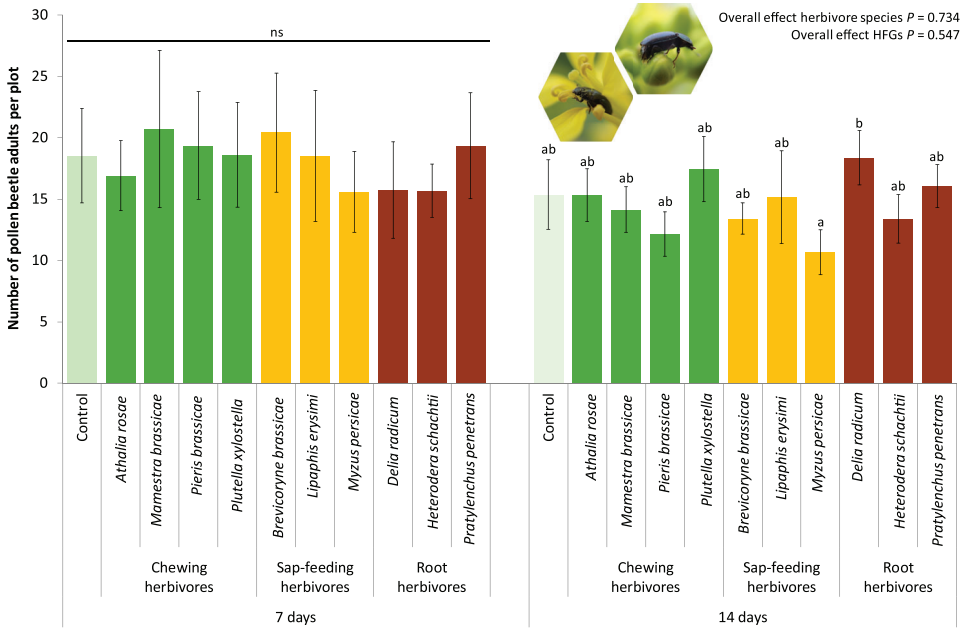

Fig. 3 Number of pollen beetle (*Meligethes aeneus*) adults recorded (mean ± SE) on uninfested plots (control) of *Brassica nigra* and on plots of plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs): chewing, sap-feeding or root herbivory. Observations were made at two time points: 7 and 14 days after infestation. Number of replicates per herbivore treatment varied between 10 and 14. Letters above bars indicate significant differences at $P \leq 0.05$ based on least squares means tests for 14 days after infestation. Pictures show a pollen beetle adult resting in the flower (left) and a female laying an egg inside a flower bud (right).

adult pollen beetles and larval performance, we recorded 4,355 adult beetles and recovered 1,582 larvae from flower buds. In general, plant exposure to root, sap-feeding and chewing herbivores did not influence pollen beetle (*Meligethes aeneus*) adult preference, but influenced larval performance. On average, about three adult beetles were observed per plant. Pollen beetle adults had no clear preference for herbivore-infested or control plants (Fig. 3; Table A2 and A3). Fewer pollen beetles colonized plants infested with the aphid *M. persicae* compared with plants infested with the root herbivore *D. radicum* (Fig. 3, Tukey's *post hoc* test, $P = 0.043$), and a similar trend was observed for plants infested with sap-feeding herbivores when compared with plants infested with root herbivores (Tukey's *post hoc* test, $P = 0.071$), but only when plants had been flowering for 14 days. Abundance of pollen beetle adults did not vary with time. Similar numbers of adult pollen beetles were recorded at 7 and 14 days since the plants started flowering (Fig. 3, Table A2 and A3). On average, about 17 pollen beetle larvae were recovered per plant. We found similar numbers of pollen beetle larvae in flower buds of infested and control plants, irrespective of larval instar (Fig. A5, Table A4). The average weight of all larvae found per plant was 0.66 ± 0.44 (SD) mg. First instar larvae weighed on average 0.19 ± 0.08 (SD) mg, second instar larvae 0.84 ± 0.38 (SD) mg. Pollen beetle larvae performed differently on flower buds of infested and control plants (Fig. A6, table A4), and this depended on beetle larval instar (Fig. A6, Table A4). First instar pollen beetle larvae performed better on buds of plants infested with the nematode *H. schachtii* compared with buds of plants infested with the nematode *P. penetrans* (Fig. A6, Tukey's *post hoc* test, $P < 0.010$), or caterpillars *P. xylostella* (Fig. A6, Tukey's *post hoc* test, $P < 0.010$), or *M. brassicae* (Fig. A6, Tukey's *post hoc* test, $P = 0.027$), and tended to perform better on buds of plants infested with the nematode *H. schachtii* compared to buds of uninfested plants (Fig. A6, Tukey's *post hoc* test, $P = 0.054$). Second instar pollen beetle larvae performance was affected by herbivory (Fig. A6, Table A4), but *post hoc* groups could not be identified.

Effect of herbivore infestation on plant seed set

Individual *B. nigra* plants produced on average about 11,000 seeds. Plants set different numbers of seeds depending on whether plants had or had not been exposed to different herbivores (Fig. 4, Table A5). The strength of this effect depended on the position of the plant within a plot (Table A5). When regarding the seed set of central plants, plants exposed to root herbivores set more seeds than plants exposed to chewing herbivores (Fig. 4, Tukey's *post hoc* test, $P = 0.012$), and we observed the same trend when comparing plants exposed to root herbivores and uninfested plants (Fig. 4, Tukey's *post hoc* test, $P = 0.093$). Plants infested with *P. xylostella* caterpillars set fewer seeds when compared with plants infested with the root-feeding larvae *D. radicum* (Fig. 4, Tukey's *post hoc* test, $P < 0.010$) or plants infested with *L. erysimi* aphids (Fig. 4, Tukey's *post hoc* test, $P = 0.021$),

and tended to set fewer seeds than plants infested with *H. schachtii* nematodes (Fig. 4, Tukey's *post hoc* test, $P = 0.081$). None of the individual herbivore treatments differed statistically from uninfested plants (Fig. 4). Seed set of side plants of a plot was not influenced by herbivore exposure (Table A5).

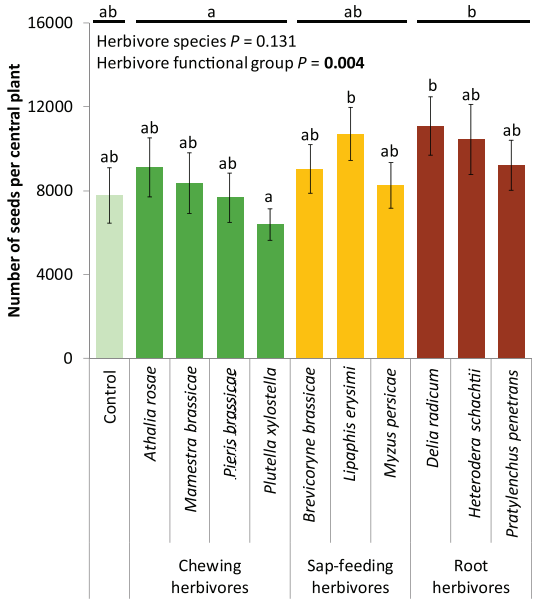

Fig. 4 Number of seeds (mean ± SE) of central plants of uninfested plants (control) of *Brassica nigra* and plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs): chewing, sap-feeding or root herbivory. Number of replicates per herbivore treatment varied between 14 and 16. Letters above bars indicate significant differences at $P \leq 0.05$ based on Tukey's *post hoc* tests.

Discussion

Our data show that attack of *B. nigra* plants by a range of herbivore species differently affected the interactions of these plants with mutualist pollinators and an antagonist florivore. Pollinator community composition was affected by herbivory, whereas overall pollinator attraction was maintained. The composition of the pollinator community visiting flowers of uninfested plants differed from that visiting flowers of chewing and root herbivore-infested plants. Main responders in the pollinator communities to changes induced by herbivory were syrphid flies, bumblebees, and solitary bees. Fewer syrphid flies were attracted to herbivore-infested plants than to undamaged plants. In addition, syrphid flies tend to spent more time on flowers of root herbivore-infested plants compared with flowers of plants infested with sap-feeding herbivores. Although the preference of pollen beetle adults was not affected by herbivory, beetle larvae performed best on plants infested with the nematode *H. schachtii*. The changes in pollinator community composition and syrphid fly visitation can explain the observed increase in seed set of root herbivore-infested plants.

Our data suggest that some of the ecological and plant fitness consequences of herbivore-

induced plant responses depend both on the feeding site or guild of the attacker, and thus identify specificity in plant-mediated interactions between herbivores and pollinators. Plant responses to chewing and root herbivores affected pollinator community composition and pollinator visitation, whereas responses of plants to sap-feeding herbivores did not. Plant responses to sap-feeding herbivores are mainly mediated by the salicylic acid (SA) phytohormonal pathway, whereas plant responses to chewing and root herbivores are mainly mediated by the jasmonic acid (JA) phytohormonal pathway (Walling 2000, Ali and Agrawal 2012, Erb et al. 2012). Our data show that plant responses mediated by JA more frequently lead to changes in pollinator attraction and visitation if compared with plant responses mediated by SA. Plant exposure to chewing and root herbivores seem to affect pollinator groups differently, suggesting different plant responses to chewing and root herbivory. Specificity in other plant-mediated interactions, such as herbivore-parasitoid, or herbivore-herbivore interactions, is often found for herbivore feeding guilds. For example, non-host chewing herbivores differentially affected parasitoid behaviour compared with non-host sap-feeding herbivores (de Rijk et al. 2016). Aphid infestation can increase colonization and performance of chewing herbivores (Soler et al. 2012), but caterpillar infestation reduced the performance of different sap-feeding herbivores (Stout et al. 1997). The observed effects in herbivore-pollinator interactions suggests specificity of plant responses, and both the feeding guild and site of herbivores characterise such specificity, *i.e.* differential induction of flower traits by chewing and sap-feeding, and above- and belowground herbivores.

In addition to specificity of effect between inducers, receivers in plant-mediated herbivore-pollinator interactions might also show specificity in response to the induced plant. Indeed, we observed different responses of syrphid flies, bumblebees and solitary bees to herbivore attack. Differential responses of pollinators to herbivore attack led to differences in the composition of the pollinator communities visiting flowers of plants under attack. This has also been shown for various other annual brassicaceous plants (Hoffmeister et al. 2016). Differential responses of pollinators to herbivore attack can be explained by the use of different subsets of flower traits (Junker et al. 2013), and differences in the ability to learn (Bitterman et al. 1983, Daly et al. 2001, Raine and Chittka 2008, Riffell 2011). Pollinator species differ in their innate preferences between and within flower traits (Sutherland et al. 1999, Cook et al. 2003, Junker and Blüthgen 2010, Junker et al. 2013), as well as the flower traits they use for associative learning (Schiestl and Johnson 2013, Knauer and Schiestl 2015). Thus, for some pollinators herbivore-induced changes might result in "unfamiliar" floral cues, or flowers with "undesirable" rewards, and hence the flowers will not be visited (Kessler and Baldwin 2007, Kessler et al. 2008, Kessler and Halitschke 2009). Some pollinators can quickly learn to associate visual and odour cues to rewards provided by plants (Schiestl and Johnson 2013, Knauer and Schiestl 2015), and this might maintain pollinator visitation

if only visual and odour cues are affected by herbivory and rewards remain of good quality. Thus, due to differential responses of pollinators to herbivore-induced changes in flower traits, plants can maintain overall pollinator attraction and compensate for attack by different herbivore species.

Plants might compensate for herbivory by maintaining pollinator attraction, or increase the attraction of pollinators which deliver better pollination service. In the present study, plants compensated for herbivory; despite damage caused by herbivores, infested plants produced similar numbers of seeds as uninfested plants. Compensation in terms of seed set in response to herbivory in Brassicaceae seems to be common (Poveda et al. 2005, Lucas-Barbosa et al. 2013, Schiestl et al. 2014, Hoffmeister et al. 2016). Interestingly, in our study, *B. nigra* plants overcompensated in seed set for herbivory by root herbivores. As direct effects of root herbivores on seed set are often negative (van Dam 2009, Barber and Gorden 2015, Ghyselen et al. 2016, Johnson et al. 2016), positive effects might more likely be mediated by indirect effects, *via* plant-mediated herbivore-pollinator or herbivore-herbivore interactions. It could be that the pollinator community associated with root herbivore-infested plants delivers better pollination. This community was composed of more bumblebees than found in other plants. More bumblebee visits may increase the amount of pollen that flowers receive, or the quality of the pollen mixture deposited (Aizen and Harder 2007, Rader et al. 2009, Garibaldi et al. 2013), resulting in higher seed production (Silander and Primack 1978). In addition, syrphid flies tended to spend more time on root-herbivore infested plants, which could result in a larger amount of pollen being deposited (Conner et al. 1995). Unfortunately, our data is unsuitable to quantify the relation between pollinator visitation and seed set. In generalized pollination systems, flower visitors differ in pollination effectiveness, which includes any characteristic of a pollinator that contributes to successful pollination and seed set (Ne'eman et al. 2010). To quantify the relation between pollinator visitation and seed set, measuring pollination effectiveness is essential. Further studies should investigate pollination effectiveness of different flower visitors of *B. nigra*. Our data suggests that herbivore-induced changes in the composition of the pollinator community can influence the reproductive output of *B. nigra* plants.

The indirect effects of root herbivory on plant reproductive output *via* other community members, such as antagonist flower visitors, cannot be excluded. The pollen beetle *M. aeneus* is an abundant species on flowers of *B. nigra,* and can be considered an important flower antagonist. Herbivore infestation did not influence the adult preference of this species. Other studies addressing the effects of herbivory on antagonistic flower visitors found decreased preference for, and reduced damage on herbivore-infested plants by the second herbivore (McCall 2006, McArt et al. 2013). Artificial damage to flowers can increase (Gorden 2013) or reduce (Boyer et al. 2016) the preference of florivores for damaged plants. The contrasting results by this and other studies might be explained by

the different florivores investigated, which use different traits to identify and utilize host plants, and are thus differentially affected by herbivore-induced plant responses. Adult pollen beetles use vision and scent to identify host plants (Jönsson et al. 2007, Doering et al. 2012, Piesik et al. 2013); traits which have been shown to change upon exposure of plants to herbivores (Schiestl et al. 2014, Lucas-Barbosa et al. 2016). Furthermore, pollen beetle adults can probably detect changes in host-plant quality that result from herbivore infestation (Ekbom and Borg 1996, Ekbom 1998, Hervé et al. 2014, Hervé et al. 2016). According to the preference-performance hypothesis (Thompson 1988), selection will only favour adult pollen beetles that make a distinction between uninfested and infested plants if the performance of the larvae is affected. Pollen beetle larvae performed best on plants infested with the nematode *Heterodera schachtii*. Flower traits that determine pollen beetle larval performance, such as pollen quality or the nutritional value of other flower tissues (Cook et al. 2004, Hervé et al. 2016) could have been influenced by plant responses to *H. schachtii*. However, pollen beetle females prefer buds of specific sizes for oviposition, and oviposition in the field may be more restricted by availability of resources than by their quality (Ekbom and Borg 1996). Dependence of the pollen beetle on resource quantity over quality excludes possibilities for plant-mediated interactions, and is a plausible explanation for the lack of plant-mediated herbivore-herbivore interactions between various herbivores and pollen beetles adults as observed in the present study. Still, many herbivores do respond to changes in plant quality (Stam et al. 2014) and root herbivory has been shown to increase plant resistance to leaf-chewing herbivores *via* the systemic induction of defensive compounds in the aboveground parts of the plant (Soler et al. 2005, Van Dam et al. 2005, van Dam 2009). Further studies are needed to identify if root herbivory affects plant seed set *via* resistance to other herbivores, not monitored in our study.

Our study shows specificity of plant-mediated herbivore - flower-visitor interactions that arises from herbivore- and functional-group-specific-induced plant responses as well as differential responses of flower-visitor species to herbivore-induced plants. Although *B. nigra* plants maintained reproductive output despite these indirect interactions between mutualists and antagonists, feeding by herbivores from specific functional groups may enhance reproductive output. These results indicate that indirect plant-mediated interactions are likely reflected in natural selection on plant growth, defence and reproductive strategies (Poelman and Kessler 2016).

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Chapter 4

Plant ontogeny determines strength and associated plant fitness consequences of plant-mediated interactions between herbivores and flower visitors

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Submitted

Abstract

Plants show considerable ontogenetic variation in growth-defence strategies to maximize reproductive output within a community context. Most work on plant ontogenetic variation in growth-defence trade-offs has focussed on interactions with antagonistic insect herbivores. Plants respond to herbivore attack with phenotypic changes. Despite the knowledge that plant responses to herbivory affect plant mutualistic interactions with pollinators required for reproduction, such indirect interactions between herbivores and pollinators have not been included in the evaluation of how ontogenetic growth-defence trajectories affect plant fitness. In a common garden experiment with the annual *Brassica nigra*, we investigated whether exposure by various herbivore species on different plant ontogenetic stages (vegetative, bud, or flowering stage) affects plant flowering traits, interactions with flower visitors, and results in fitness consequences for the plant. Our results show that plant interactions with flower visitors such as pollinators and pollen beetles (*Meligethes* spp.) are affected by plant exposure to herbivores in all three plant ontogenetic stages tested. The outcome of herbivore - flower-visitor interactions was specific for the combination of herbivore species and plant ontogeny. Plant exposure to herbivores resulted in either positive or negative effects on pollinator visitation (attraction, number of flowers visited, time spent per visit and per flower) and generally reduced pollen beetle numbers compared to its abundance on undamaged plants. Plant exposure to herbivores in the vegetative or bud stage most strongly affected flower visitors, while plant exposure to herbivores in the vegetative stage also affected plant flowering traits and reproductive output. Especially exposure of vegetative plants to *Brevicoryne brassicae* aphids and *Pieris brassicae* caterpillars negatively affected inflorescence number and seed production. The indirect interaction web between herbivores and flower visitors is dynamic and varies over plant ontogeny. Consequences of herbivory for plant reproductive output are strongest when plants are attacked by herbivores early in life. Such differences in selection pressures imposed by herbivores to specific plant ontogenetic stages likely drive the evolution of distinct ontogenetic trajectories in growth-defence-reproduction strategies and include indirect interactions between herbivores and pollinators.

Keywords: *Brassica nigra*, florivores, herbivore-induced plant responses, indirect interactions, ontogenetic variation, ontogenetic trajectories, plant defence, plant reproduction, pollinators

Introduction

Interactions between species form the foundations of ecological communities. The role that individuals of a species play within a community can show considerable ontogenetic variation (Miller and Rudolf 2011, Nakazawa 2015). For example, juvenile stages of most amphibians and some insects live in aquatic environments, but after metamorphosis to the adult stage, they move to terrestrial environments. Throughout ontogeny, the individuals that are part of different food webs connect aquatic and terrestrial ecosystems by ontogenetic shifts in habitat (Knight et al. 2005). Other examples are ontogenetic diet shifts of aquatic predators that connect pelagic and benthic systems (Rudolf and Rasmussen 2013), and metamorphosis of larval herbivorous insects to adult mutualist pollinators that connects herbivore and pollinator networks associated with plants (Bronstein et al. 2009, Revilla and Encinas–Viso 2015, Ke and Nakazawa 2018, Smith et al. 2018). It is now well recognised that the ontogenetic stage of individuals has considerable impact on community structure and dynamics (Miller and Rudolf 2011, Nakazawa 2015), but how these community processes in turn feed-back to fitness of individual community members is less well understood.

Over their lifetime, plants gradually develop through various ontogenetic stages; from cotyledon and seedling stage, to pre-reproductive and reproductive stage, and eventually to over-maturation and death (Boege and Marquis 2005). During each of these stages, plants interact with different community members such as antagonistic herbivores, competing plants, or beneficial pollinators. To maximize their fitness, plants may display ontogenetic variation in resistance to herbivores (Boege and Marquis 2005, Barton and Koricheva 2010), investment in growth to outcompete neighbours for light (Zhang et al. 2008, Tonnabel et al. 2017), or investment in the recruitment of natural enemies of herbivores to reduce the impact of herbivore attack (Quintero et al. 2013, Quintero et al. 2014). Distinct ontogenetic trajectories in growth-defence strategies may allow plants to optimize responses to shifts in the cost-benefit balance of growth-defence trade-offs that arise from the dynamic nature of communities that plants are part off (Barton and Boege 2017). An important implication of ontogenetic growth-defence trajectories is that investment in one component may not only energetically trade-off against other components of the trajectory, but may also alter the plant's interaction with other community members (Lucas-Barbosa 2016, de Vries et al. 2017). For example, large plants may be more apparent to herbivores as a result of increased investment in growth induced by competition for light (de Vries et al. 2019). Beneficial organisms required for successful reproduction have not been included in theoretical frameworks of growth-defence trade-offs through plant ontogeny, despite the fact that plant responses to herbivory can affect plant interactions with flower visitors with consequences for plant reproduction (Chautá et al. 2017, Rusman et al. 2018 - chapter 3).

Herbivores may affect flower visitors in various ways and this can be modulated by plant ontogeny. Herbivores may directly affect flower visitation by physically repelling pollinators (Canela and Sazima 2003), or by removing flower biomass that makes plants less attractive to flower visitors (Sõber et al. 2010). Effects of herbivores may be even more extensive through plant-mediated (indirect) interactions between herbivores and pollinators that arise from plant responses to herbivory (Kessler and Halitschke 2009, Lucas-Barbosa 2016). Herbivore-induced changes in expression of flower traits affect the visitation of mutualistic and antagonistic flower visitors (McArt et al. 2013, Hoffmeister et al. 2016, Stam et al. 2018, Rusman et al. 2018 - chapter 3). Plant ontogeny will most likely modulate how herbivores affect flower visitors. The costs of herbivory vary over plant ontogeny, as well as responses of plants to herbivory (Boege et al. 2007, Rostás and Eggert 2008, Diezel et al. 2011, Brütting et al. 2017), and these extend to effects on flower abundance and other traits that influence pollinators (Desurmont et al. 2015, Hoffmeister et al. 2016). Therefore, we expect ontogenetic variation in plant-mediated herbivore - flower-visitor interactions. Because flower visitors directly interact with the reproductive organs of the plant, variation in visitation by flower visitors comes with plant fitness consequences (Cariveau et al. 2004). So far, ontogenetic variation in indirect herbivore - flower-visitor interactions and associated fitness consequences have not been investigated.

In this study on the annual plant *Brassica nigra*, we investigated whether exposure of plants at different ontogenetic stages to various herbivore species affects plant flowering traits, interactions with flower visitors, and results in fitness consequences for the plant. In a manipulative experiment, we exposed plants in the vegetative, bud, or flowering stage to one of six herbivore species. More specifically, we studied whether herbivore attack to plants at these three ontogenetic stages affects i) plant phenological traits and flower abundance, ii) visitation rates of mutualists (pollinators), iii) abundance of antagonists (florivorous pollen beetles, *Meligethes* spp.), and iv) seed production. Knowledge on ontogenetic variation with respect to the effects of herbivory on plant reproduction will elucidate whether there is variation in selection pressures imposed by herbivores to plants at different ontogenetic stages that potentially drive the evolution of plant defence through their ontogeny (Poelman and Kessler 2016, Barton and Boege 2017, Ochoa-López et al. 2018).

Materials and Methods

Plant and insects

For our experiments, we used Black mustard (*Brassica nigra*) accession CGN06619 that originates from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands) and was propagated by open field pollination. Seeds were germinated in trays. One-week-

old plants were transplanted to and cultivated in pots (Ø 17 cm – 2 L) filled with potting soil (Lentse potgrond) and sand in a 1:1 volume ratio under greenhouse conditions (23 ± 2 °C, 50 -70 % r.h., L16:D8). Two-week-old plants were transferred to an outside area protected by insect screen. Three-week-old plants were transplanted into the field.

We exposed plants to six herbivore species; two chewing herbivores (larval *Pieris brassicae* and *Athalia rosae*), two sap-feeding herbivores (adult *Brevicoryne brassicae* and *Lipaphis erysimi*), and two root-feeding herbivores (larval *Delia radicum* and *Heterodera schachtii*). The caterpillar *P. brassicae* and the aphids *B. brassicae* and *L. erysimi* originated from the surroundings of Wageningen (The Netherlands), and they are routinely reared in the Laboratory of Entomology (Wageningen University) under greenhouse conditions (22 ± 1 °C, 50 -70 % r.h., L16:D8). *Pieris brassicae* and *B. brassicae* were reared on Brussels Sprouts (*Brassica oleracea* variety *gemmifera* cultivar Cyrus); *L. erysimi* was reared on *Raphanus sativus*. The sawfly *A. rosae* originated from surroundings of Würzburg (Bavaria, Germany). The larvae were reared on *Raphanus sativus* under greenhouse conditions (22 ± 1 °C, 50 - 70 % r.h., L16:D8). The cabbage root fly *D. radicum* originated from Zeewolde (The Netherlands). Larvae were reared on turnips (*Brassica rapa*) or rutabaga (*Brassica napus*) in a climate cabinet (22 ± 1 °C, 50 - 70 % r.h., L16:D8). Cysts of the nematode *H. schachtii* originated from the rearing of the Institute for Rational Sugar production (IRS) (Bergen op Zoom, The Netherlands). The population used was IRS 07-01-04.02 and originates from Woensdrecht, The Netherlands. Nematodes were reared on rapeseed (*B. napus* cultivar Jennifer). Cysts were hatched in the laboratory using a 3 mM $ZnCl_2$ solution. After hatching, nematodes were flushed out of the hatching sieve using tap water, and solutions containing about 1000 nematodes (J2 stage) in 4 mL of water were used to infest plants.

Common garden experiment - Field design

A common garden experiment was designed to investigate whether herbivore infestation of plants at different developmental stages affected flowering traits (number of inflorescences and phenological traits), flower visitors (mutualistic pollinators and antagonistic pollen beetles, *Meligethes* spp.), and plant seed production. We planted 160 plots of *B. nigra* in a field of the experimental farm of Wageningen University, The Netherlands. Plots were organized in 10 rows and 16 columns, and each plot was composed of five plants - one central plant and four plants surrounding the central plant - at a distance of 20 cm. Distance between central plants of neighbouring plots was 1.5 m. Each day 24 plots were planted, except for day 7, when we planted 16 plots. Plots of columns 1-8 were planted between day 1 and 4, column 5 was kept empty, and plants of columns 9-16 were planted between day 4 and 7. Treatments were randomly assigned over plots using a Latin square design, *i.e.* each combination of herbivore species and

plant developmental stage never occurred twice in the same row or column. Treatments were equally divided over the planting dates and replicated eight times.

Plant treatments

Plants were infested with herbivores at different developmental stages, either in the vegetative, bud, or flowering stage (Fig. 1). Plots in the vegetative stage were infested one day after planting. Plots in the bud or flowering stage were infested one day after three out of the five plants of a plot had reached the bud or flowering stage, including the central plant. We considered that a plant had reached the bud stage when buds of the first flowering stalk rose above the leaves. Plants were considered flowering when the first flower opened. We placed a mesh tent (95 l x 95 w x 190 h cm) for 24 h over each plot for infestation, to provide the necessary time for the herbivores to settle on the plants. Uninfested control plots were also covered with a mesh tent for 24 h right after planting, when all 5 plants were still in the vegetative stage.

To infest *B. nigra* plants with aboveground herbivores, we placed 10 first instar chewing herbivores or 10 adult sap-feeding herbivores on two true leaves (five per leaf). For the root herbivore *D. radicum* we placed 10 first instar larvae at the base of the stem. To infest a plant with nematodes, 4 mL of solution containing about 1000 nematodes was added in four holes (1 mL per hole) around the main stem of the plant. Such holes were approximately 10 cm deep with a diameter of 0.5 cm, and were made at 2 cm from the stem of each of the five plants (Rusman et al. 2018 - chapter 3).

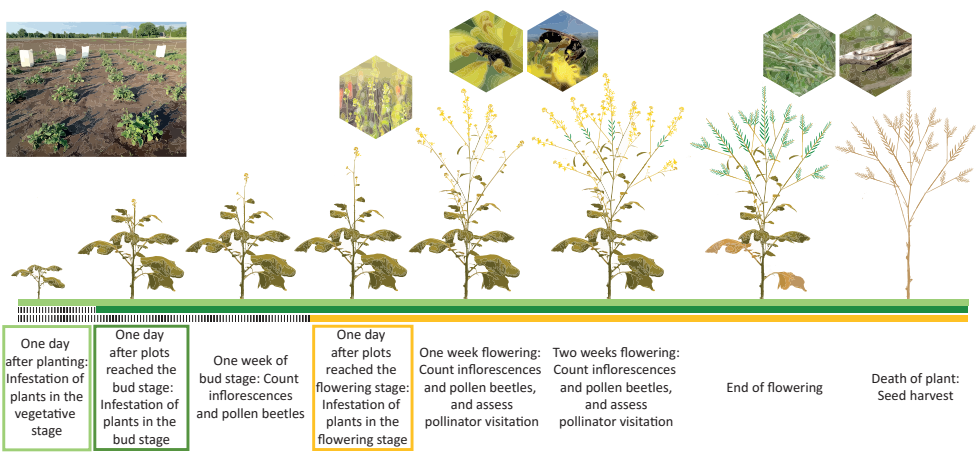


Fig. 1 Schematic representation of the timeline of the experiment. All *Brassica nigra* plants were planted at the same time in the field. Plants exposed to herbivores in the vegetative stage (light green bar) were infested one day after planting. Plants exposed to herbivores in the bud stage (dark green bar) were infested one day after plots had reached the bud stage. Plants exposed to herbivores in the flowering stage (yellow bar) were infested one day after plots had reached the flowering stage.

We recorded survival of all aboveground herbivore species, per plant of a given plot, at six days after infestation. When two or fewer herbivores were recorded, the plant was re-infested with five individuals of second-instar chewing larvae or five adult sucking herbivores. Root samples were taken from approx. 80 plants at the end of the experiment, and checked for damage by *D. radicum* or nematode presence.

Effect of herbivore infestation and plant ontogeny on flower abundance and phenological traits

To investigate if flower abundance and several phenological traits of *B. nigra* were influenced by herbivore exposure at different plant developmental stages, we recorded flower abundance and followed plant development of infested and uninfested plants. Flower abundance was assessed by counting all inflorescences of each plant at three time points (Fig. 1): a) 7-9 days after plants had entered the bud stage, and at b) 7-9 and c) 14-16 days after plants had started flowering. Plant phenological traits were assessed by monitoring plant development daily. We recorded the start of the bud stage, the start of the flowering stage, and the end of flowering. Plants were considered finished with flowering when all buds and flowers were gone, and only developing and ripe siliques remained on the flower stalks. We calculated the duration of the bud stage by subtracting the number of days needed to reach the bud stage from the number of days needed to reach the flowering stage. We calculated the duration of the flowering stage by subtracting the number of days needed to reach the flowering stage from the number of days to the termination of flowering.

Effects of herbivore infestation and plant ontogeny on floral mutualists and antagonists

To investigate if pollinator visitation to *B. nigra* flowers was influenced by herbivore exposure of plants at different developmental stages, we recorded pollinator behaviour in plots of infested and uninfested plants at two time points during the flowering stage: a) between 7 and 9 days of flowering and b) between 14 and 16 days of flowering (Fig. 1). Pollinator visitation to a plot was monitored for 10 minutes. When a pollinator entered the plot and had contact with a flower, identity of the pollinator, number of flowers visited, and time spent per flower were recorded (Rusman et al. 2018 - chapter 3). If during the observation of a pollinator other pollinators entered the plot, their visitation and identity was recorded as well. If the same pollinator individual returned to the plot under observation after having visited a different plot, we scored that visit as a new visit. Pollinator identity was recorded as belonging to one of six groups: honeybees (*Apis mellifera*), bumblebees (*Bombus terrestris, Bombus lapidarius, Bombus pratorum*, *Bombus hortorum*, and other *Bombus* spp.), syrphid flies (several *Eristalis* spp. and several other syrphid species), solitary bees (several *Andrena* and *Lasioglossum* species but also other

Apidae excluding *Bombus* spp.), other flies (non-syrphid Diptera), and butterflies (*Pieris* spp. and other Lepidoptera). Observations were stored in a handheld computer (Psion Workabout Pro^{tm} 3, London, UK) programmed with The Observer XT software (version 10, Noldus Information Technology, Wageningen, The Netherlands). Observations were carried out between 9 am and 1 pm, or 2 pm and 5 pm, when weather conditions were suitable for pollinator activity (15 - 30^{o} C and wind speed ≤ 6 m·s^{-1}).

To investigate if pollen beetle (*Meligethes* spp.) colonization was influenced by plant exposure to herbivores in different plant developmental stages, we monitored pollen beetle abundance on plots of infested and uninfested plants. Pollen beetle abundance was monitored by counting the number of adult pollen beetles on each plant of a given plot at three time points: a) between 7 and 9 days after plots had entered the bud stage, and b) between 7 and 9 and c) between 14 and 16 days after plots had entered the flowering stage (Fig. 1). Observations were carried out between 2 pm and 6 pm when weather conditions were suitable for pollen beetle activity (15 - 30^{o} C and wind speed ≤ 6 m·s^{-1}).

Effects of herbivore infestation at different plant ontogenetic stages on plant seed production

To investigate if life-time seed production was influenced by herbivory during different plant developmental stages, we assessed seed number and biomass of the same plants used for the experiments where pollinator behavioural observations and pollen beetle abundance were monitored. We harvested seeds of three plants for each plot; the central plant and two side plants (randomly selected and not adjacent to each other). First harvesting date for each plant was selected before the first siliques would lose their seeds (Rusman et al. 2018 - chapter 3). At this time point, we harvested all ripe siliques. Immature siliques and flowers were left on the plant and harvested whenever they were ripe, up to one month later. We stored the seeds in paper bags at room temperature in a dry storage room until seeds were processed. We calculated total number of seeds per plant by weighing 100 seeds, and the total weight of seeds harvested per plant. We estimated the total number of seeds by dividing total seed weight by the weight of 100 seeds and multiplied the result by 100. The weight of one seed was estimated by dividing the weight of 100 seeds by 100.

Statistical analysis

For count data such as the number of insects, flowers, days and seeds, we used generalized linear (mixed) models with a Poisson distribution and a log link function, or negative binomial distribution with a log link function to correct for overdispersion. We ran two models: The first model included herbivore treatment and plant developmental stage and their interactions as fixed factors. In the case of numbers of insects or flowers, this

model was run for each time point separately. The second model included herbivore treatment and time point (except for phenological traits or seeds) and their interactions as fixed factors. This model was run for each plant developmental stage separately. Interactions were removed from the models if they were statistically non-significant ($P > 0.05$). For *post hoc* analyses we used Tukey's *post hoc* tests. Random factors were selected using a backward approach; all random factors such as day (not for phenological traits or seeds), time (morning vs afternoon; only for pollinators), plot (not for pollinators), plant position (not for pollinators), day*treatment (not for phenological traits or seeds), were added to the model and removed if they explained less than 0.03 of the variation or were statistically non-significant ($P > 0.05$). We used the lme4 (Bates et al. 2015), multcomp (Hothorn et al. 2008), and lsmeans (Lenth 2016) packages for these analyses. For continuous data such as time spent per visit and per flower by pollinators, we used linear (mixed) models with a Gaussian distribution and identity link function or a Gamma distribution with a log link function if the data did not follow a normal distribution. The same fixed factors, random factor selection approach, and software packages as for count data were used. We analysed pollinator community composition by comparing the pollinator community composition of infested and uninfested plots with a Chi-square test. Expected pollinator community composition was calculated by summing pollinators within each group for all plots and dividing this number by the total number of pollinators. This results in an expected percentage for each pollinator group. This percentage was then multiplied by the total number of pollinators recorded for infested or uninfested plots (Rusman et al. 2018 - chapter 3). We did not take the pollinator community composition of uninfested plots as expected pollinator community composition because the pollinator community distributes over the different treatments including the uninfested plots based on pollinator preference in the choice situation, e.g. the community composition of the uninfested plots is affected by the presence of the infested plots (Rusman et al. 2018 - chapter 3). If pollinator community composition was explained by plant exposure to herbivores, pair-wise comparisons among all herbivores within one plant developmental stage were performed, and pair-wise comparisons among the three plant developmental stages for each herbivore species were performed using Chi-square tests. To correct for multiple tests of pair-wise comparisons, we adjusted the *P*-values using the false discovery rate (FDR) correction. We used the fifer package for these analyses (Fife 2014). In addition, to assess which pollinator groups contributed to differences between herbivore species and plant developmental stages, we calculated the standardized residuals for each pollinator group in each treatment and used a threshold value of ± 2 (Sharpe 2015, Rusman et al. 2018 - chapter 3). Pollinator groups which composed less than 1% of the community were excluded from the analysis, e.g. other flies (0.3%), butterflies (0.04%). For correlations between the number of inflorescences and insects, we computed the correlation coefficient r using the Pearson or Kendall

method, depending on the distribution of the data. Correlation graphs were made using the ggpubr package (Kassambara 2018). All analyses were carried out in R (version 3.4.3 × 64, 2017, The R Foundation for Statistical Computing Platform).

Results

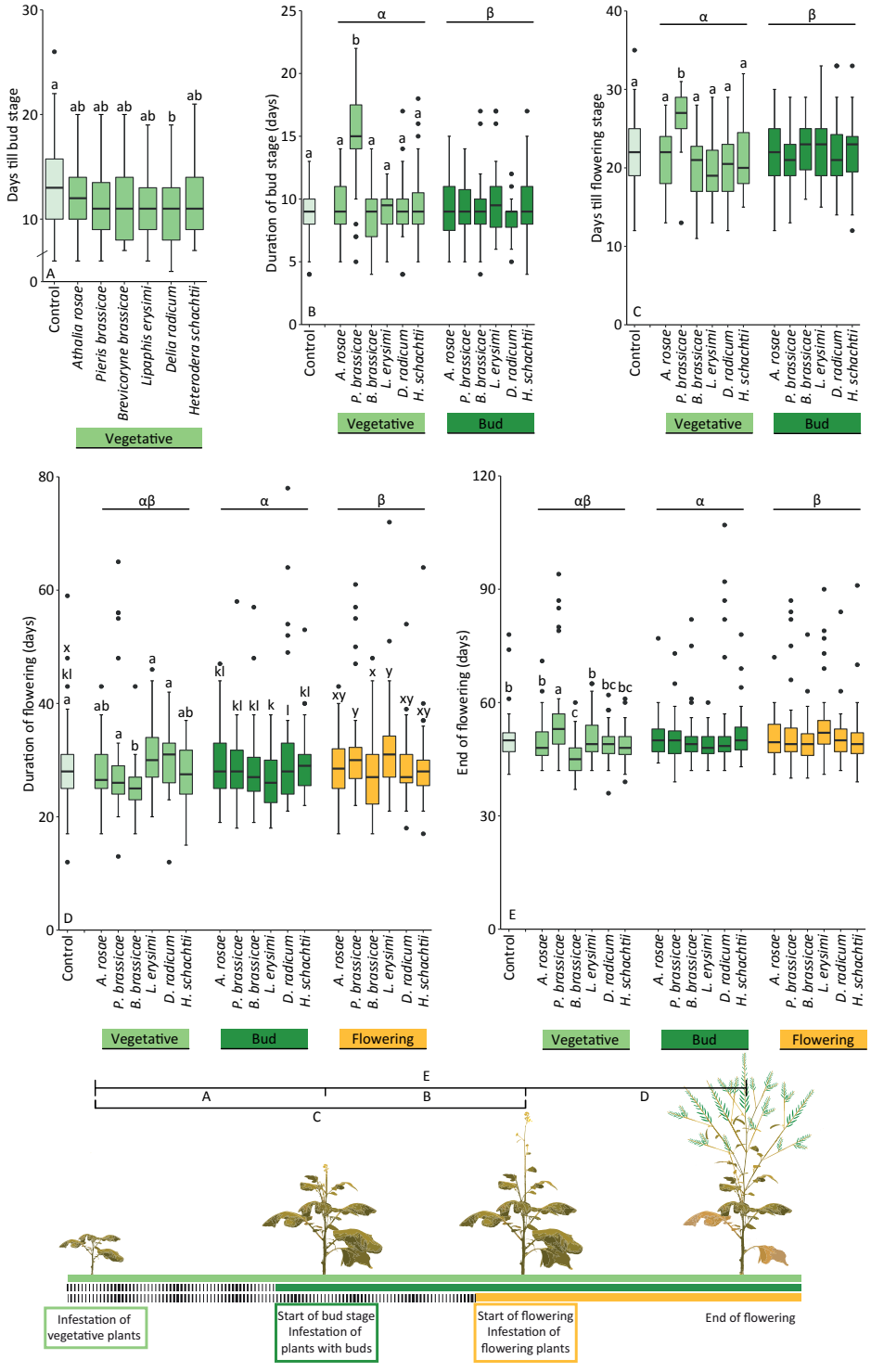
Effects of herbivore infestation and plant ontogeny on plant phenological traits

Once transplanted into the field, it took plants on average 12 days to reach the bud stage and an additional 10 days to reach the flowering stage. Plants flowered for an average of 29 days. Plant exposure to herbivores affected all plant phenological traits (Fig. 2, Appendix B Table B1). The ontogenetic stage in which plants were exposed to the herbivores influenced the effects on plant phenological traits: herbivore infestation in the bud stage led to shorter flowering period compared with plants infested in the flowering stage (Tukey's *post hoc* test, $P = 0.048$). Compared with plants infested in the vegetative stage, herbivore infestation in the bud stage led to a shorter bud stage (Tukey's *post hoc* test, $P < 0.001$), fewer days to reach the flowering stage (Tukey's *post hoc* test, $P < 0.001$), and earlier termination of flowering (Tukey's *post hoc* test, $P = 0.005$). Effects of individual herbivores varied over plant ontogeny, especially for the herbivores *P. brassicae*, *B. brassicae*, and *L. erysimi* (Table B2). For plants exposed in the vegetative stage, we observed herbivore-species-specific effects of *P. brassicae* and *B. brassicae* for the duration of the bud stage, days till flowering, and termination of flowering (Fig. 2). Herbivore-species-specific effects on flowering time were observed for plants exposed in all plant ontogenetic stages (vegetative, bud, and flowering).

Effects of herbivore infestation and plant ontogeny on flower abundance

Plant exposure to herbivores at different ontogenetic stages and the herbivore's identity affected the number of flowers produced by *B. nigra*. Plant exposure to herbivores affected flower abundance on two out of the three time points on which flower abundance was recorded, *i.e.* at one week after plants had started to produce buds, and two weeks after plants had started flowering (Fig. 3, Table B3 + B4). One week after plants had started to produce buds, plants exposed to herbivores in the vegetative stage had fewer inflores-

Fig. 2 (right) Phenological traits of uninfested *Brassica nigra* plants and plants infested with one of six herbivore species at different plant ontogenetic stages. We monitored when plants reached the bud stage (A), duration of the bud stage (B), when plants reached the flowering stage (C), duration of flowering (D), and end of flowering (E). Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Number of replicates per herbivore treatment varied between 33 and 45 plants, and between 76 and 78 for uninfested plants. Letter groups (a - d, k - n, w - z) above bars indicate significant differences ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests.


Days till bud stage
Duration of bud stage (days)
Days till flowering stage
Duration of flowering (days)
End of flowering (days)
Control
Athalia rosae
Pieris brassicae
Brevicoryne brassicae
Lipaphis erysimi
Delia radicum
Heteroderа schachtii
A. rosae
P. brassicae
B. brassicae
L. erysimi
D. radicum
H. schachtii
Vegetative
Bud
Flowering
A
B
C
D
E
Infestation of vegetative plants
Start of bud stage Infestation of plants with buds
Start of flowering Infestation of flowering plants
End of flowering

cence on display compared with plants exposed to herbivores in the bud stage (Tukey's *post hoc* test, $P < 0.001$), and this effect was particularly strong for plants exposed to *P. brassicae* caterpillars (Tukey's *post hoc* test, $P < 0.001$). Effects of herbivore species on the number of inflorescences were always negative, and observed for plants exposed to *P. brassicae* caterpillars in the vegetative stage and *H. schachtii* nematodes in the bud stage one week after plants had started to produce buds (Fig. 3). These negative effects were still apparent two weeks after plants had started flowering for plants exposed to *P. brassicae* caterpillars in the vegetative stage, and by this time, plants exposed to *B. brassicae* aphids in the vegetative stage also had fewer inflorescences compared to uninfested plants. Effects of *P. brassicae, B. brassicae*, and *L. erysimi* on the number of inflorescences two weeks after plants had started flowering varied depending on the ontogenetic stage in which the plant was attacked (Table B2).

Effects of herbivore infestation and plant ontogeny on floral mutualists – pollinator community

The four most abundant pollinator groups that visited *B. nigra* flowers were honeybees (86%), syrphid flies (11%), bumblebees (2%), and solitary bees (1%). Plant exposure to herbivores affected overall pollinator community composition (Fig. B1, χ^2 test, $\chi^2 = 75.30$, df = 54, $P = 0.025$). Effects of individual herbivore species on pollinator community composition varied over plant ontogeny (Table B2). For example, pollinator community composition differed for plants exposed to *D. radicum* in the bud stage compared to plants exposed to these root-feeding herbivores in the vegetative stage (χ^2 test, $P = 0.004$) or flowering stage (χ^2 test, $P = 0.006$). We found herbivore-species-specific effects on pollinator community composition for plants exposed to herbivores in the bud stage (χ^2 test, $\chi^2 = 29.15$, df = 18, $P = 0.047$), but pairwise testing only revealed marginally insignificant differences between herbivores (Fig. B1). All pollinator groups responded to the herbivore treatments and their responses were specific for timing of herbivore induction during plant ontogeny (Fig. B1, Table B5). For instance, solitary bees visited plants infested with the root herbivore *D. radicum* in the bud stage less frequently compared to the expected community, but bumblebees visited these plants more frequently. In contrast, solitary bees visited plants infested in the flowering stage with the root herbivore *D. radicum* more frequently compared to the expected community, but bumblebees visited these plants less frequently (Table B5).

Effects of herbivore infestation and plant ontogeny on floral mutualists – pollinator attraction

One week after plants had started flowering, plots were on average visited by 32 pollinators which visited 89 flowers during the 10 min observation time, and two weeks after plants had started flowering this increased to 44 pollinators and 101 flower visits. Plant exposure to herbivory affected the number of pollinators visiting plots and the

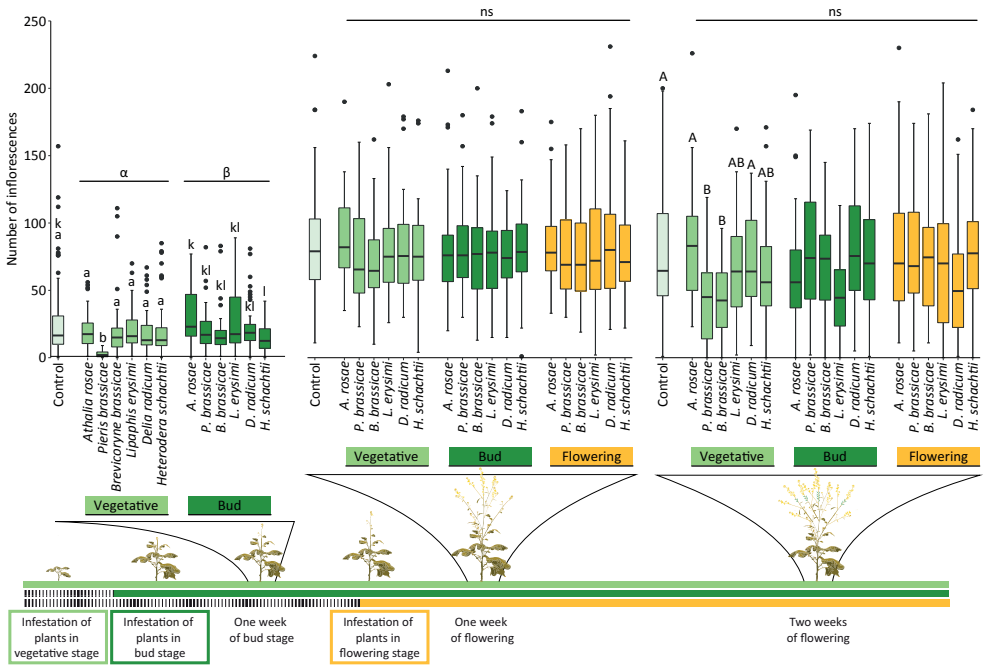


Fig. 3 Number of inflorescences of uninfested *Brassica nigra* plants and plants infested with herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Plants were monitored for the number of inflorescences at three time points: between 7 - 9 days after plants had reached the bud stage, and between 7 - 9 days, and 14 - 16 days after plants had started flowering. Number of replicates per herbivore treatment varied between 28 and 45 plants, and between 76 and 78 for uninfested plants. Letter groups (a - d, k - n) above bars indicate significant differences ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests, and small or capital letters were used for different time-points. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

number of flowers visited by pollinators for both time points; one and two weeks after the start of flowering (Fig. 4, Fig. B2 - B4 and Fig. B7 - B9, Tables B3 and B4). Herbivore exposure differently affected pollinator attraction and flower visitation depending on the ontogenetic stage in which plants had been exposed to the herbivores. One week after plants had started flowering, plants exposed to herbivores in the bud stage were visited by a larger number of pollinators, especially honeybees, compared with plants exposed to herbivores in the flowering stage (Tukey's *post hoc* tests, total pollinators (TP): $P = 0.025$, honeybees (HB): $P = 0.034$), and effects were particularly strong when plants were exposed to larvae of the sawfly *A. rosae* (Tukey's *post hoc* tests, TP: $P = 0.025$, HB: $P = 0.034$), *L. erysimi* aphids (Tukey's *post hoc* test, TP: $P = 0.007$), or *H. schachtii* nematodes (Tukey's *post hoc* tests, TP: $P < 0.001$, HB: , $P < 0.001$). However, plants

exposed to herbivores in the flowering stage received more syrphid-fly visits compared to plants exposed in the vegetative stage (Tukey's *post hoc* test, $P = 0.036$). In contrast to the number of pollinator visits, more flowers were visited by all pollinators for plants exposed to herbivores in the flowering stage compared to plants exposed in the bud stage one week after the start of flowering (Tukey's *post hoc* tests, TP: $P = 0.006$, HB: $P = 0.002$) and compared to plants exposed in the vegetative stage on both time points (Tukey's *post hoc* tests, 1-week: TP: $P < 0.001$, HB: $P < 0.001$, 2-weeks: TP: $P < 0.001$, HB: $P = 0.005$).

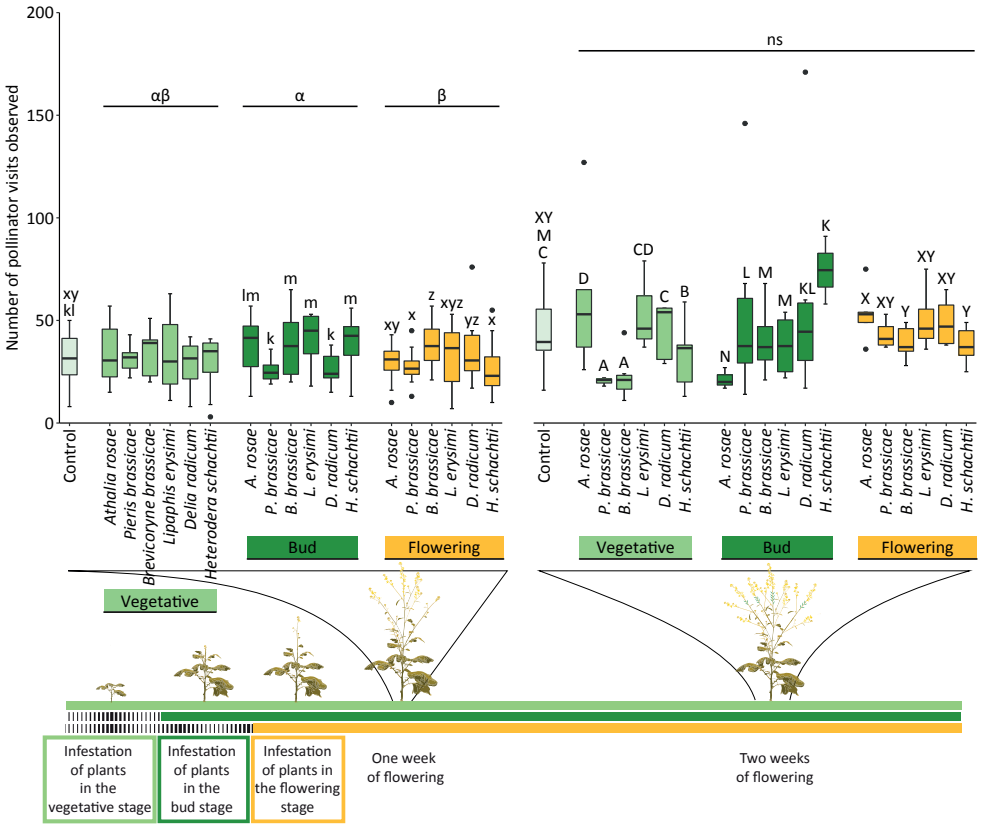


Fig. 4 Number of pollinator visits observed on flowers of uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 - 9 days, and 14 - 16 days after plots had started flowerings. For 7 - 9 days after plots started flowering, the number of replicates per herbivore treatment varied between 7 and 9, and was 16 for uninfested plants. For 14 - 16 days after plots started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 10 for uninfested plants. Letters groups (a - d, k - n, w - z) above bars indicate significant differences at ($P <$ 0.05) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests, and small or capital letters were used for different time-points. Greek letters above lines indicate significant differences ($P <$ 0.05) between plant developmental stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

This was true for four out of six herbivore species (Table B2). The effects of individual herbivore species on the number of total pollinators, honeybees, and syrphid flies varied with plant ontogeny for both time points (Table B2). Herbivore-species-specific effects were observed for plants exposed in the vegetative stage (number of flowers visited by syrphid flies (SF)), bud stage (number of TP, HB, and SF, and number of flowers visited by TP, HB, and SF), and flowering stage (number of TP and HB, and number of flowers visited by SF) for both time points (Fig. 4, Fig. B2 - B4 and Fig. B7 - B9, Tables B3 and B4).

The total number of pollinators positively correlated with the number of inflorescences one and two weeks after the start of flowering (Fig. B5 and B6, r = 0.25, t = 3.19, df = 157, $P = 0.002$; r = 0.49, t = 5.08, df = 82, $P < 0.001$ respectively). In both cases, plant exposure to herbivores affected this correlation (Fig. B5 and B6). One week after plants had started flowering, we only found a positive correlation between the number of inflorescences and the total number of pollinators for plants exposed in the flowering stage to herbivores (r = 0.49, t = 3.85, df = 46, $P < 0.001$), especially to *L. erysimi* aphids (r = 0.79, t = 3.19, df = 6, $P = 0.019$) and the root herbivore *D. radicum* (r = 0.85, t = 3.95, df = 6, $P = 0.008$). In contrast, we only found a positive correlation for plants exposed to herbivores in the vegetative stage two weeks after the start of flowering stage (r = 0.79, t = 6.75, df = 28, $P < 0.001$). Due to low number of observations two weeks after the start of flowering, we could only analyse each herbivore species for all three plant ontogenetic stages combined. We found a positive correlation between the number of inflorescences and the total number of pollinators for uninfested plants (r = 0.76, t = 3.29, df = 8, $P = 0.011$), and plants exposed to the sawfly *A. rosae* (r = 0.66, t = 2.79, df = 10, $P = 0.019$) or the nematode *H. schachtii* (r = 0.74, t = 3.62, df = 11, $P = 0.004$).

Effects of herbivore infestation and plant ontogeny on floral mutualists – visitation times and flower visits

During pollinator visitations, plant exposure to herbivores influenced the number of flowers visited per visit (for HB and SF), the time spent per flower (for HB and SF), and visitation duration (for SF) (Fig. B10 - B15, Tables B3 and B4). Herbivore infestation differently affected pollinator visitation depending on the ontogenetic stage in which plants were exposed to the herbivores. One week after plants had started flowering, syrphid flies visited more flowers per visit on plants exposed to herbivores in the flowering stage compared to plants exposed in the bud stage (Tukey's *post hoc* test, $P = 0.044$), and this was especially true for plants exposed to larvae of the sawfly *A. rosae* (Tukey's *post hoc* tests, $P = 0.044$) or the nematode *H. schachtii* (Tukey's *post hoc* tests, $P = 0.039$). Syrphid flies spent more time per flower on plants exposed to herbivores in the bud stage compared to plants exposed in the vegetative stage (Tukey's *post hoc*

tests, $P = 0.002$), whereas honeybees spent more time per flower on plants exposed in the vegetative stage compared to plants exposed in the flowering stage (Tukey's *post hoc* tests, $P = 0.033$). In the case of honeybees, this effect was also observed two weeks after plants had started flowering (Tukey's *post hoc* tests, $P = 0.020$), but then honeybees also visited fewer flowers of plants that had been exposed to herbivores in the vegetative stage compared to plants exposed in the flowering stage (Tukey's *post hoc* tests, $P = 0.015$). Effects of individual herbivore species on the visitation behaviour of honeybees and syrphid flies varied over plant ontogeny (Table B2). Herbivore-species-specific effects were observed for plants exposed in the vegetative stage (visitation duration SF, number of flowers visited by HB and SF), bud stage (number of flowers visited by SF, time spent per flower by HB), and flowering stage (visitation duration and time spent per flower by SF, number of flowers visited by HB and SF) for one week after plants had started flowering (Fig. B10 - B15, Tables B3 and B4). At two weeks after plants had started flowering, we collected a limited number of observations for syrphid flies and could not analyse the effects of plant exposure to different herbivore species on their visitation behaviour. For honeybees, herbivore-species-specific effects on the number of flowers visited per visit and the time spent per flower were observed for plants exposed in the vegetative and flowering stage, and for number of flowers visited per visit also for plants exposed in the bud stage (Fig. B11 and B12).

Effects of herbivore infestation and plant ontogeny on a floral antagonist

We observed on average one pollen beetle adult per plant one week after plants had started to produce buds; this number increased to an average of seven beetles per plant one week after plants had started flowering, and declined to about three beetles per plant two weeks after plants had started flowering. On all monitored time points, plant exposure to herbivores affected the number of pollen beetle adults observed per plant (Fig. 5, Tables B3 and B4) and the effect depended on the ontogenetic stage in which plants were exposed. Plants exposed in the vegetative stage contained fewer adult beetles compared to plants exposed in the bud stage one week after plants had started to produce buds (Tukey's *post hoc* test, $P = 0.035$) and one week after plants had started flowering (Tukey's *post hoc* test, $P = 0.019$). Interestingly, this was true for four out of six individual herbivore species one week after plants had started to produce buds, and for the other two herbivore species one week after plants had started flowering (Table B2). Effects of the herbivores *A. rosae*, *P. brassicae*, and *B. brassicae* were always negative, and observed for plants exposed in the vegetative stage (one week after plants had started to produce buds, and one week after plants had started flowering), and the bud stage (one week after plants had started to produce buds, and two weeks after plants had started flowering), but not for plants exposed in the flowering stage (Fig. 5, Tables B3 and B4).

The number of pollen beetle adults per plant positively correlated with the number of inflorescences per plant for all three time points: one week after plants had started to produce buds (Fig. B16, $\tau = 0.46$, $z = 32.73$, $P < 0.001$), and one and two weeks after the start of flowering (Fig. B17 and B18, $\tau = 0.45$, $z = 33.87$, $P < 0.001$; $\tau = 0.53$, $z = 37.46$, $P < 0.001$ respectively). We found a positive correlation between the number of inflorescences and the number of pollen beetle adults for all treatments combined, for plants exposed in different ontogenetic stages to herbivores, and for each herbivore species and plant ontogenetic stage combination (Fig. B16 - B18).

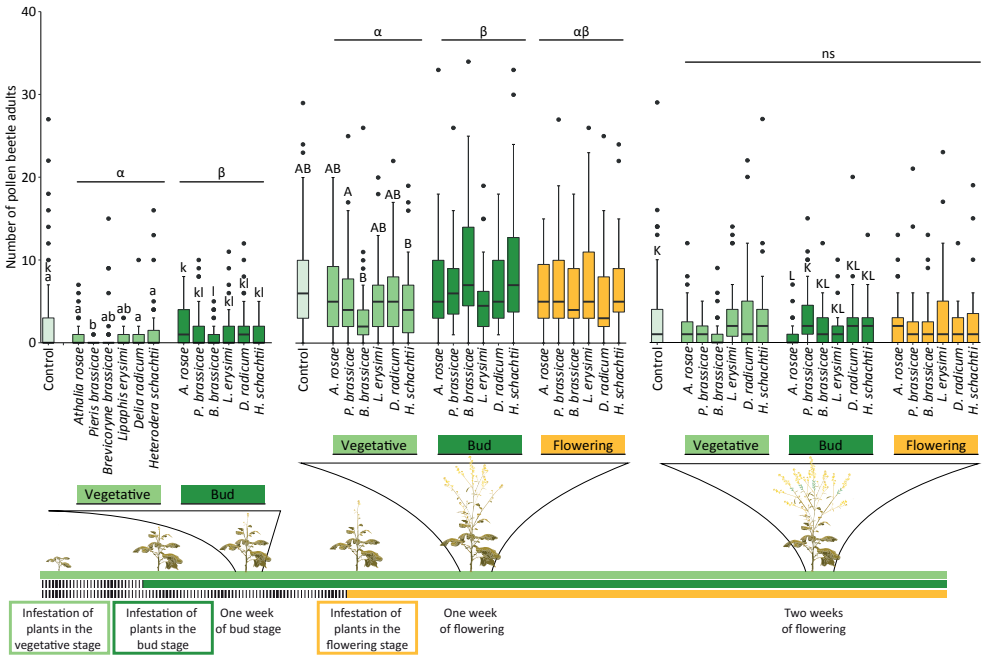


Fig. 5 Number of pollen beetle adults (*Meligethes* spp.) observed on flowers of uninfested (control) *Brassica nigra* plants and plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Plants were monitored for the number of pollen beetle adults at three time points: between 7 - 9 days after plants had reached the bud stage, and between 7 - 9 days, and 14 - 16 days after plants had started flowering. Number of replicates per herbivore treatment varied between 28 and 45 plants, and between 76 and 78 for uninfested plants. Letters groups (a - d, k - n) above bars indicate significant differences at ($P <$ 0.05) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests, and small or capital letters were used for different time-points. Greek letters above lines indicate significant differences at ($P <$ 0.05) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

Effects of herbivore infestation and plant ontogeny on plant seed production

Brassica nigra plants produced on average 10,000 seeds, with an average individual seed weight of 1 mg. Plant exposure to herbivores affected plant seed set and seed weight (Fig. 6 and B19, Table B6). Effects of herbivory on seed set of side plants in a plot depended on the ontogenetic stage in which plants had been exposed to the herbivores. Plants exposed to herbivores in the vegetative stage produced fewer seeds than plants exposed to herbivores in the flowering stage (Tukey's *post hoc* test, $P < 0.010$). This was especially true for plants exposed to *B. brassicae* in the vegetative stage if compared with plants that were exposed to these aphids in the flowering stage (Tukey's *post hoc* test, $P < 0.001$) but also in the bud stage (Tukey's *post hoc* test, $P < 0.001$). For the average seed set per plant per plot and seed weight, the effect of individual herbivore species varied with plant ontogeny (Table B2). This was also the case for seed set of the central plants. Herbivore-species-specific effects were restricted to plants exposed in the vegetative stage for both seed set and weight (Fig. 6 and B19). We observed a positive effect of infestation with *L. erysimi* aphids on seed set, and negative effects on seed set by infestation with *P. brassicae* caterpillars and *B. brassicae* aphids. The aphid *B. brassicae* also had a negative effect on seed weight.

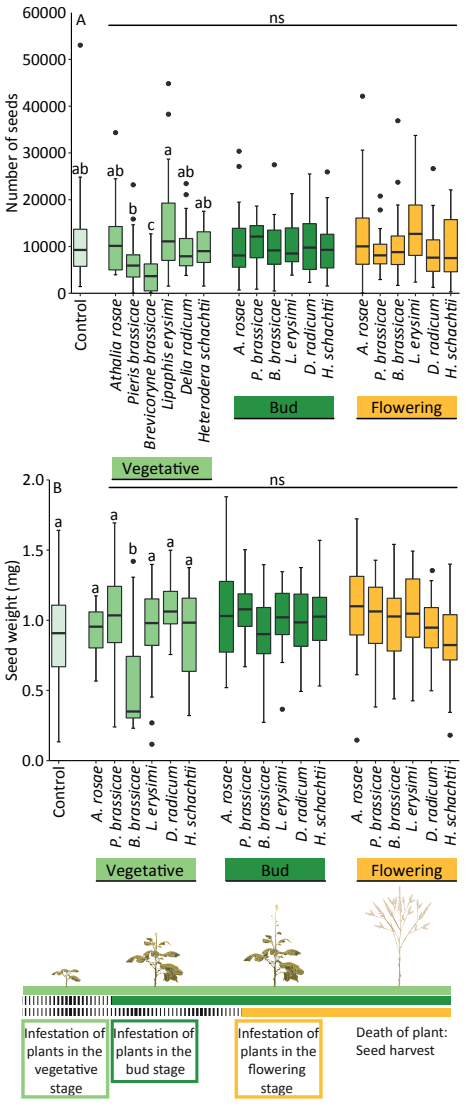


Fig. 6 Number of seeds (A) and seed weight (B) of seeds produced by uninfested (control) *Brassica nigra* plants and plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Number of seeds and seed weight were averaged for 3 plants per plot (1 central plant and 2 side plants). The number of replicates per herbivore treatment varied between 43 and 50, and between 88 and 91 for uninfested plants. Letters above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant developmental stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

Discussion

The findings of our study illustrate how herbivore encounter with plants at different ontogenetic stages and the herbivore's identity affect flowering traits, interactions with pollinator mutualists and flower-feeding antagonists, and eventually plant reproductive output. Exposure to herbivores in all three plant ontogenetic stages tested (vegetative, bud and flowering) influenced plant interactions with pollinators, especially honeybees and syrphid flies, and pollen beetles (*Meligethes* spp.), and the outcome was specific for the combination of herbivore species and plant ontogeny. Plant exposure to herbivores resulted in either positive or negative effects on pollinator visitation (attraction, number of flowers visited, time spent per visit and flower) and generally reduced pollen beetle abundance compared to numbers on undamaged plants. Effects of herbivory on flower visitors were most prominent when plants were exposed to herbivores in the vegetative or bud stage. Plant exposure to herbivores in the vegetative stage especially affected flowering traits and plant seed production. Especially plant exposure to *B. brassicae* aphids and *P. brassicae* caterpillars negatively affected inflorescence number and seed production when plants were exposed to these herbivores in the vegetative stage. Both plant ontogeny and herbivore identity shaped the effects of herbivory on plant flowering traits, the outcome of indirect interactions with flower visitors, and plant fitness.

Our data show that plant ontogeny is a major determinant of indirect interactions between herbivores and flower visitors. Plant-mediated interactions were specific for the identity of the interaction partners (herbivores and flower visitors) and the direction of the interactions – positive, negative, or neutral – varied with plant ontogeny. The resulting indirect interaction web between herbivores and flower visitors appears dynamic and variable over plant ontogeny. Ontogenetic variation in indirect interaction webs is evident from aquatic systems (predatory fish and pelagic and benthic food webs), and systems which include both aquatic and terrestrial components (amphibians or aquatic insects) (Miller and Rudolf 2011, Nakazawa 2015), but has received limited attention in plant-insect systems (Waltz and Whitham 1997). Ontogenetic variation in indirect herbivore-flower-visitor interactions can be explained by two non-mutually exclusive mechanisms: different plant responses over plant ontogeny or varying effects of herbivory on plants determined by the timing of herbivore encounter. Indeed, plant responses to herbivory vary with plant ontogeny (Rostás and Eggert 2008, Diezel et al. 2011, Ochoa-López et al. 2015), which includes changes in flower traits (Desurmont et al. 2015, Hoffmeister et al. 2016). The timing of events such as herbivory determines the strength of interactions between herbivores and plants, and subsequent effects on flower visitors (Chase 2003, Vannette and Fukami 2014, Stam et al. 2018). Herbivory during the vegetative stage of a plant may be costlier as compared to herbivory during the flowering stage, due to increased investments in resistance and loss of important photosynthetic tissues early in life (Boege et al. 2007, de Vries et al. 2018, 2019). This may lead to reduced investments in

flowers because of resource limitations (Quesada et al. 1995, Strauss et al. 1996, Poveda et al. 2003, Poveda et al. 2005). Herbivory affects organisms that engage in interactions with the plant under attack soon after the event, and new interactions established over the rest of the life of the plant are affected until the plant dies (Stam et al. 2018). Hence, herbivore attack will echo through the indirect interaction web differentially depending on the arrival time of herbivores.

Whereas herbivore infestation in various plant ontogenetic stages affected plant-mediated interactions with flower visitors, effects of herbivory on plant flowering traits were most pronounced when herbivores colonized plants early in life, *i.e.* during their vegetative stage. Different effects of herbivore infestation on the plant and on flower visitors suggest the importance of other traits than flower abundance for interactions with flower visitors. Indeed, variation in flower traits such as flower scent and morphology, which we did not assess in this study (but see Rusman et al. 2019 - chapter 5), may explain part of the variation in flower-visitor communities (Kuppler et al. 2016, Soper Gorden and Adler 2016). Interestingly, plant exposure to herbivores affected the correlation between the number of inflorescences and the number of pollinator mutualists, but not the correlation between the number of inflorescences and the number of antagonist pollen beetles. This may indicate differential importance of resource quantity and quality for mutualists and antagonists (Cariveau et al. 2004, Wenninger et al. 2016). Floral antagonists may prefer/better assess resource quantity than quality (Ekbom and Borg 1996, Althoff et al. 2013, Wenninger et al. 2016, Rusman et al. 2018 - chapter 3), whereas both may be important for floral mutualists during foraging (Kuppler et al. 2016). Still, we found cases where herbivore exposure did not affect the number of inflorescences but did affect pollen-beetle colonization. This could be due to changes in traits that determine flower apparency, such as floral volatiles, which floral antagonists use to locate resources (Jönsson et al. 2007, Theis and Adler 2012). This suggests that antagonist-antagonist interactions are not limited by the dependence of antagonists on resource quantity (Rusman et al. 2018 - chapter 3). Differential importance of resource quantity and quality for mutualists and antagonists may have important fitness consequences for the plant because herbivory differentially affects flower quantity and quality, and mutualists and antagonists differentially affect plant reproduction (Grass et al. 2018, Soper Gorden and Adler 2018).

We show that fitness consequences of herbivory can be specific to the plant ontogenetic stage that is attacked. Herbivory only affected plant reproduction when plants were colonized by herbivores early in life, while still in the vegetative stage. This indicates that the potential trade-off between plant growth/reproduction and defence is limited to herbivore attack in specific plant developmental stages (Lucas-Barbosa et al. 2011, Lucas-Barbosa 2016). Differences in fitness consequences of herbivory through plant ontogeny can result from direct effects such as allocation costs or developmental

constraints (Barton and Boege 2017), and from indirect effects *via* plant-mediated interactions (Strauss 1997, Lucas-Barbosa 2016, Poelman and Kessler 2016). For annual plants, the main defence strategy early in plant development is resistance, while later in plant development this switches to tolerance (Boege et al. 2007). Increased investment of black mustard in resistance and loss of important photosynthetic tissues due to herbivore damage early in life will be especially costly (de Vries et al. 2018, 2019). This can explain our observed reduction in flowers and seed set for plants exposed in the vegetative stage to *P. brassicae* caterpillars or *B. brassicae* aphids. Alternatively, expression of the most effective defence strategy against *P. brassicae* and *B. brassicae* may be limited early in plant development due to developmental constraints (Quintero et al. 2013, Barton and Boege 2017) or the absence of natural enemies of herbivores early in the season (Mira and Bernays 2002, Gómez-Marco et al. 2016). Herbivory affects seed production also indirectly *via* interactions with plant-associated antagonists and mutualists (Strauss et al. 2002, McArt et al. 2013, Pashalidou et al. 2015). The complete network of indirect plant-mediated interactions will determine the consequences for plant reproduction (Poelman and Kessler 2016, Soper Gorden and Adler 2018, Rusman et al. 2018 - chapter 3). Hence, ontogenetic variation in the indirect interaction web between herbivores and flower visitors may have contributed to the observed fitness consequences of herbivory. This is important for understanding plant evolution because ontogenetic variation in indirect interaction webs and plant reproduction will alter patterns of diffuse (co) evolution (Strauss et al. 2005, Agrawal et al. 2012, Bassar et al. 2012, Guimarães Jr et al. 2017). Aside from the underlying mechanisms, ontogenetic variation in consequences of herbivory for plant reproduction likely generates stage-specific herbivore selection pressures, which drive the evolution of defence ontogenetic trajectories (Barton and Boege 2017, Ochoa-López et al. 2018).

Plant ontogeny is important for direct and indirect consequences of herbivory. Therefore, studies on the evolution of plant defences need to consider ecologically relevant timing of herbivory. Plants can be particularly vulnerable to specific herbivores during certain stages in life, and herbivores that arrive on plants in specific ontogenetic stages can generate particularly strong selection pressures. Some plant traits can be effective anti-herbivore defences during specific plant developmental stages, but mediate ecological costs of herbivory in other plant development stages (Barton and Boege 2017). The adaptive value of traits can therefore only be assessed when considering the complete life cycle of the organisms, and their interactions based on ecologically relevant timing. By determining direct and indirect interactions, ontogeny creates developmental-stage-specific communities which may have profound effects on overall community structure and dynamics (Miller and Rudolf 2011, Nakazawa 2015). Moreover, community structure and dynamics may affect trait evolution (Siepielski and Benkman 2004, Agrawal et al. 2012, Utsumi et al. 2013, Guimarães Jr et al. 2017), resulting in eco-

evolutionary dynamics driven by ontogenetic variation (Ohgushi 2016).

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Chapter 5

Floral plasticity: Herbivore-species-specific-induced changes in flower traits with contrasting effects on pollinator visitation.

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Plant, Cell & Environment, *In press*

Abstract

Plant phenotypic plasticity in response to antagonists can affect other community members such as mutualists, conferring potential ecological costs associated with inducible plant defence. For flowering plants, induction of defences to deal with herbivores can lead to disruption of plant-pollinator interactions. Current knowledge on the full extent of herbivore-induced changes in flower traits is limited and we know little about specificity of induction of flower traits and specificity of effect on flower visitors. We exposed flowering *Brassica nigra* plants to six insect herbivore species and recorded changes in flower traits (flower abundance, morphology, colour, volatile emission, nectar quantity, and pollen quantity and size) and the behaviour of two pollinating insects. Our results show that herbivory can affect multiple flower traits and pollinator behaviour. Most plastic floral traits were flower morphology, colour, the composition of the volatile blend, and nectar production. Herbivore-induced changes in flower traits resulted in positive, negative or neutral effects on pollinator behaviour. Effects on flower traits and pollinator behaviour were herbivore species-specific. Flowers show extensive plasticity in response to antagonist herbivores, with contrasting effects on mutualist pollinators. Antagonists can potentially act as agents of selection on flower traits and plant reproduction *via* plant-mediated interactions with mutualists.

Keywords: *Brassica nigra* (black mustard), flower colour, flower morphology, flower rewards, flower volatiles, herbivore-induced plant responses, phenotypic plasticity, plant defence, plant-mediated interactions, specificity

Introduction

Plants interact with an incredibly diverse community of plant mutualists and antagonists. Antagonists range from large mammals to tiny insects, and microscopic bacteria and viruses. Each of these attackers may differ in the mode of attack as well as the fitness costs associated with the attack. To successfully defend against this plethora of attackers, plants evolved various defensive strategies (Agrawal 2011, Karban 2011, Dicke and van Loon 2014). These strategies include inducible defences that allow resistance to be fine-tuned to the specific attacker and save metabolic costs of resistance in the absence of herbivores (Karban and Baldwin 1997, Karban 2011, Kessler 2015). However, such phenotypic plasticity can affect many other interactions between the plant and its environment in addition to the target herbivore, with potential negative effects on plant fitness, imparting so called ecological costs (Heil 2002, Strauss et al. 2002, Poelman 2015, Poelman and Kessler 2016). Ecological costs of phenotypic plasticity are most clearly revealed in flowering plants. The majority of flowering plants are involved in one or more intimate interactions with pollinators, and disruption of plant-pollinator interactions can be directly detrimental for plant fitness (Wilcock and Neiland 2002, Ollerton et al. 2011). A number of studies has identified disruptions in plant-pollinator interactions due to plant responses to insect herbivores (Liao et al. 2013, Schiestl et al. 2014, Hoffmeister et al. 2016), with consequences for plant reproduction (Botto-Mahan et al. 2011, Chautá et al. 2017, Rusman et al. 2018 - chapter 3).

Herbivore-induced changes in pollinator visitation are mediated by plasticity in flower traits. Plants attract pollinators through various flower traits: flower abundance, size, morphology, colour, volatiles, and rewards (nectar and pollen) (Junker and Parachnowitsch 2015, Akter et al. 2017). Flower traits are highly plastic and change readily in response to environmental factors, such as herbivory (Strauss 1997, Lucas-Barbosa et al. 2011). Changes in response to herbivory include most flower traits involved in pollinator attraction (Bruinsma et al. 2014, Cozzolino et al. 2015, Hoffmeister et al. 2016, Lucas-Barbosa et al. 2016). Herbivore-induced changes in floral traits may vary considerably depending on herbivore species (Pareja et al. 2012, Rusman et al. 2018 - chapter 3). In our previous work we identified that herbivore-pollinator interactions may depend on feeding guild and site of the herbivore as well, but we did not characterize which flower traits were responsive to herbivory (Rusman et al. 2018 - chapter 3). Thus, limited knowledge is available of the full extent to which different flower traits are affected by herbivore induction, the specificity of induction of flower traits, and specificity of effects on flower visitors. We think that knowledge on the specificity of herbivore-induced plant responses provides an opportunity to explore the extent to which plasticity in floral traits supports or leads to disruption of plant-pollinator interactions.

We expect flower plasticity to follow patterns of specificity in inducible defences known

for foliar plant responses. Specificity in inducible defences is to some extent mediated by phytohormones, which are involved in defence as well as in reproduction. Chewing herbivores, for instance, mainly induce the jasmonic acid (JA) pathway, whereas sap-feeding herbivores usually suppress JA and/or induce the salicylic acid (SA) pathway (Ali and Agrawal 2012, Erb et al. 2012, Thaler et al. 2012). Root-feeding herbivores induce the JA pathway, but the phytohormonal network seems different belowground compared to aboveground, resulting in different plant responses (Johnson et al. 2016). Flower traits are also regulated by these phytohormones. For example, SA regulates flowering time (Martínez et al. 2004) and is involved in flower formation (Rivas-San Vicente and Plasencia 2011) and flower thermogenesis (Raskin et al. 1987). Likewise, JA is involved in general developmental processes of flowering (Yuan and Zhang 2015), and regulates the expression of various flower traits (Brioudes et al. 2009, Avanci et al. 2010, Radhika et al. 2010, Muhlemann et al. 2014). Moreover, flower and defence traits are linked *via* shared genetic or biochemical pathways, *via* shared resources (Jacobsen and Raguso 2018), or *via* functional responses, where flower traits are involved in defence as well. Flowering plants use floral volatiles to attract pollinators but also natural enemies of herbivores (Lucas-Barbosa et al. 2014, Schiestl et al. 2014), and whereas pigments colour the flower, they are also toxic for herbivores (Gronquist et al. 2001). Because of the multiple links between flower and defensive traits, we expect similarities in specificity of herbivore induction.

In this study, we tested whether responses of the annual *Brassica nigra* to feeding by various herbivores affects multiple flower traits and whether herbivore-induced changes in flower traits affect pollinator behaviour. We specifically studied how herbivore-induced plant responses affect (i) flower abundance, size and morphology, (ii) flower chemistry, such as colour and odours, (iii) flower rewards, including nectar and pollen quantity and pollen size, (iv) the behaviour of two pollinating insects, the butterfly *Pieris brassicae* and the syrphid fly *Episyrphus balteatus*. We hypothesized that herbivores from similar feeding guilds/sites induce more similar changes in flower traits compared to herbivores from different feeding guilds/sites, which is predicted by specificity of elicitation of phytohormones involved in defence regulation (Ali and Agrawal 2012, Thaler et al. 2012, Johnson et al. 2016), and responses of pollinator functional groups to herbivory (Rusman et al. 2018 - chapter 3).

Materials and Methods

Plants and insects

Black mustard (*Brassica nigra* L., accession CGN06619) seeds were obtained from field open-pollinated plants and originated from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands). Seeds were germinated in trays and one-week-old plants

were transplanted and cultivated in pots (∅ 17 cm – 2 L) filled with potting soil (Lentse potgrond) and sand in a 1:1 volume ratio under greenhouse conditions (23 ± 2 ° C, 50 -70 % r.h., L16:D8). Once plants started flowering (five/six weeks old), they were used for the experiments.

We exposed plants to six herbivore species divided over three groups, here termed "herbivore functional groups" (HFGs): chewing herbivores (larvae of *Athalia rosae* L., *Plutella xylostella* L., and *Pieris brassicae* L.), sap-feeding herbivores (adults of *Brevicoryne brassicae* L. and *Lipaphis erysimi* (Kaltenbach)) and root herbivores (larvae of *Delia radicum* L.). All herbivores are specialists on Brassicaceae. The sawfly *Athalia rosae* originated from surroundings of Würzburg (Bavaria, Germany). The larvae were reared on *Raphanus sativus* under greenhouse conditions (22 ± 1 °C, 50 -70 % r.h., L16:D8). The caterpillars *P. xylostella* and *P. brassicae*, and aphids *B. brassicae* and *L. erysimi*, originated from the surroundings of Wageningen (The Netherlands), and they are routinely reared in the laboratory of Entomology (Wageningen University) under greenhouse conditions (22 ± 1 °C, 50 -70 % r.h., L16:D8). *Plutella xylostella, P. brassicae*, and *B. brassicae* were reared on Brussels sprouts plants (*Brassica oleracea* variety *gemmifera* cultivar Cyrus); *L. erysimi* was reared on *R. sativus* plants. The cabbage root fly *D. radicum* originated from St. Méloir des Ondes (Brittany, France). Larvae were reared on turnips (*Brassica rapa*) or rutabaga (*Brassica napus*) in a climate cabinet (22 ± 1 °C, 50 -70 % r.h., L16:D8). We used two different pollinator species for our experiments: The butterfly *Pieris brassicae* and the syrphid fly *Episyrphus balteatus* (De Geer). Butterflies mainly feed on nectar, and not pollen, while feeding on *B. nigra* plants. Compared to other pollinators, *P. brassicae* has a low visitation frequency in the field (Lucas-Barbosa et al. 2013, Rusman et al. 2018 - chapter 3), but may nonetheless be important for long-distance pollen dispersal (Courtney et al. 1982). Male and female *P. brassicae* butterflies were placed together to mate at one-three days after eclosing. After mating, females were provided with a 10% honey solution until being used in our behavioural experiments. *Episyrphus balteatus* pupae were obtained from Koppert Biological Systems (Berkel and Rodenrijs, The Netherlands). Adult syrphid flies were provided with sugar, pollen, water, and a Brussels sprouts plant infested with *B. brassicae* aphids, which is known to promote the development of the female reproductive system. Syrphid flies can feed on both nectar and pollen, but mainly feed on pollen of *B. nigra*. *Episyrphus balteatus* is a common flower visitor and efficient pollinator of Brassicaceae (Jauker and Wolters 2008). For both pollinators, we only used females for the behaviour experiment.

Plant treatments

We infested flowering *B. nigra* plants, one to three days from the start of flowering, by placing 10 first-instar sawfly larvae or caterpillars, or 20 adult aphids on the two lowest

bracts (5/10 per bract), or 10 first instar larvae of the root herbivore *D. radicum* at the base of the stem. Aboveground herbivores were not constrained to the bracts and free to move to their preferred feeding sites. Herbivore infestation densities were based on field observations and equalized for HFGs. After seven days of herbivore infestation, plants were used in the experiments (Chrétien et al. 2018), and various flower traits (abundance, size, morphology, colour, volatiles, nectar production, and pollen quantity and size) and pollinator behaviour were assessed. For volatile collection, insects were removed prior to the experiment. For other flower traits, we always sampled undamaged flowers and inflorescences. After experimental use, we recorded the number and instar of all aboveground herbivore species, and root samples were taken for belowground herbivore-infested and uninfested control plants to assess the actual damage caused by *D. radicum*. Control plants were kept uninfested for seven days.

Effect of herbivore infestation on flower size, morphology and colour

To investigate if flower size, morphology and colour were influenced by herbivore infestation, we measured the size, several morphological features, and the diffuse colour reflectance of flowers of plants infested with one of five herbivores (*A. rosae, P. xylostella, B. brassicae, L. erysimi,* and *D. radicum)* and uninfested plants. Whole flowers (single open uninfested flowers) were mounted on a platform made of cork (diameter 2.3 cm), located under a multispectral camera (Pixelteq SpectroCam; resolution 1.3 Mp; lens = Carl Zeiss 2.8/25 ZF-IR) at 11.4 cm. The multispectral camera was equipped with 8 filters (Appendix C Table C1). Halogen light (KL1500 fibre optic light source, Schott; Mainz, Germany) was provided from the top, next to the camera under a slight angle, from two sides. After taking a top view picture of the whole flower, the petals were separated from the rest of the flower, and a top view picture was taken from the four petals together. After multispectral image capture, the cork platform containing the petals was transferred to a spectroscopy set-up. For each petal, we measured the top (centred, 0.5 mm below the top edge of the petal) and the base (centred, 0.5 mm above the point where the petal narrows and bend downwards) with a spectrometer (SD2000, Ocean Optics; Largo, USA) using a fiber optic reflection probe and a deuterium-halogen light source (DH2000-FHS, Ocean Optics; Largo, USA) under a 162.5-degree angle at 1-2 mm. The spectrometer was calibrated using white (WS-2, TOP Sensory Systems) and black (by covering the input fiber) as reference. The diffuse reflection spectrum from 300 to 700 nm, as well as two regions of interest, the yellow/orange region (570-650 nm) and the UV region (310-370 nm), were taken as read-out. Six flowers of the final inflorescence of the top two flowering branches (three flowers per inflorescence) were measured from each plant, and flowers of six-eight plants per treatment. We processed whole flower and petal images by creating a segmentation pipeline: A supervised

Gaussian classification model was build using three images of flowers and petals for separating flower and background. All other images were segmented by this trained classification model. This was done in MATLAB (version R2017b) with the perClass toolbox (perClass Enterprise 5.2, PR Sys Design; Delft, The Netherlands). We inspected each image after automated segmentation and manually corrected the segmentation where needed in Paint (Version 6.1). Following segmentation, 42 - 48 flower images per treatment were analysed for surface area, surface area perimeter, convex area, convex area perimeter, length and width of fitting ellipse (major and minor chord length), and eccentricity (shape of fitting ellipse). With these measurements, we calculated the aspect ratio (major/minor chord length), solidity (surface area/convex area), and convexity (convex area perimeter/surface area perimeter). We analysed 164 - 196 petal images per treatment for surface area, length and width of fitting ellipse, diameter, and eccentricity of individual petals.

Effect of herbivore infestation on plant volatile emission

To investigate if volatile emission of flowering plants was influenced by herbivore infestation, we collected volatiles from plants infested with one of five herbivore species (*A. rosae, P. xylostella, B. brassicae, L. erysimi,* and *D. radicum)* and uninfested plants. We collected volatiles of the aboveground plant parts, both leaves and flowers, because it was impossible to exclude all leaves from the inflorescences. Although we cannot separate volatiles from leaves and flowers, volatiles of leaves comprise only 2% of the total volatile emission of flowering *B. nigra* plants and did not respond to herbivory in a previous study (Bruinsma et al. 2014). Thus, herbivore-induced changes in plant volatile emission of flowering plants are most likely due to changes in floral volatiles. We enclosed the aboveground plant parts in an oven bag (Toppits® Brat-Schlauch, polyester, 32 cm x 32 cm x 70 cm; Toppits, Minden, Germany). During collection, synthetic air from a gas cylinder was flushed through the bag at a flow rate of 300 mL min^{-1} by inserting a Teflon tube through an opening in the upper part of the bag, and air was sucked out (224-PCMTX8, air-sampling pump Deluxe, Dorset, UK; equipped with an inlet protection filter) at a flow rate of 200 mL min^{-1} through a second teflon tube at the opening of each bag, and volatiles were collected in a metal tube filled with Tenax-TA for 1.5 h. Collections were done in a greenhouse compartment (25 ± 1 °C, 50 -70 % r.h., L16:D8) between 12:00 and 14:00, and volatiles of six-eight plants were collected for each treatment. After collection, headspace samples were analysed using a gas chromatograph with a thermodesorption unit (GC) and coupled to a mass spectrometer (MS) (Thermo Fisher Scientific, Waltham, MA, USA). Plant volatiles were desorbed from the Tenax using a thermodesorption unit (Ultra 50:50, Markes, Llantrisant, UK) that heated the sample from 25 °C to 250 °C (5 min hold) in splitless mode at a rate of

60 °C min^{-1}. The released compounds were focused in a cold trap (ID 1.80 mm) at -0 °C, filled with Tenax and charcoal. The volatiles were transferred in splitless mode to the analytical column (30 m x 0.25 mm ID, 1 µm film thickness, DB-5, Phenomenex, Torrence, CA, USA) by flash heating the cold trap at 40 °C sec^{-1} to 280 °C (hold 10 min) and was held for 4 min at constant flow of 1 mL min^{-1}. The temperature program of the oven started at 40 °C and it immediately rose at 5°C min^{-1} to 280 °C, (4 min hold) with constant flow of 1 mL min^{-1}. An electron impact ionization at 70 eV was used to ionize the column effluent. Mass scanning was carried out from m/z 35 to 300 with 4.70 scans sec^{-1}. Compounds were putatively identified by comparing the mass spectra with the mass spectra of Wiley libraries and the Wageningen Mass Spectral Database of Natural Products. Identified compounds were confirmed based on retention index using the literature (Adams, 1995). The emission rates were only quantified for compounds that were detected in a minimum of 50% of the samples from one of the treatments, and peak area of individual compounds was divided by fresh plant biomass (g). Total ion counts were obtained to generate values for peak area and we used these values to calculate the total volatile emission of plants; a single ion was selected to generate values for peak area and used when analysing the volatile blend composition of *B. nigra* plants.

Effect of herbivore infestation on floral nectar and pollen

To investigate if floral nectar and pollen production were affected by herbivore infestation, we measured nectar and pollen quantity and pollen size of uninfested and herbivore-infested plants. Nectar was collected from eight flowers between 09:00-10:00 in the morning by using a 2 µL glass capillary (Microcaps®); three flowers of the final inflorescence of the two top flowering branches, and two flowers of the final inflorescence of the third flowering branch from the top were selected, and all flowers were two days old. Nectar of 15 plants was collected for plants infested with one of five herbivore species (*A. rosae, P. brassicae, B. brassicae, L. erysimi,* and *D. radicum*) and uninfested plants. Pollen quantity and size was measured using a flow cytometer: Multisizer II Coulter Counter (Beckman Coulter (UK) Ltd., High Wycombe, UK). The six anthers of a flower were collected in a 2 mL Eppendorf tube with 0.5 mL soap water, to prevent clumping of pollen grains. The anthers were crushed with a glass rod and then vortexed. Samples were then poured through a filter (MACS® SmartStainers, Miltenyi Biotec, Germany) with a pore size of 100 µm to get rid of debris. The filter was flushed with 6.5 mL of water to collect pollen grains stuck in the filter. We then added 13 mL of isotonic electrolyte Isoton (Isoton® II diluent, Beckman Coulter (UK) Ltd., High Wycombe, UK) and homogenized the samples prior to measurements by whirling the container around. The flow cytometer measured the number and size of all particles in 1 mL of solution. To exclude debris (crushed anther tissue etc.) we selected particles between 20 and 30

μm in our dataset, based on the particle size distribution of the samples. We multiplied the number of pollen grains in 1 mL by 20 to estimate the total number of pollen grains per flower. Pollen was collected from five flowers per plant, three flowers from the final inflorescence of the top flowering branch, and two flowers from the final inflorescence of the 2nd flowering branch from the top. All flowers used were two days old. Pollen of 10 plants was collected for plants infested with one of three herbivore species (*P. brassicae*, *L. erysimi*, and *D. radicum*) and uninfested plants.

Effect of herbivore infestation on pollinator behaviour

To investigate if pollinator behaviour was influenced by plant infestation, we recorded the behaviour of two pollinators, the butterfly *P. brassicae*, and the syrphid fly *E. balteatus*, in two choice situations. Individual pollinators were offered two plants; an uninfested plant, and a plant infested with one of five herbivore species (*A. rosae, P. xylostella, B. brassicae, L. erysimi,* and *D. radicum*). A single butterfly or syrphid fly was released at a time, at 100 cm from the plants. Each pollinator was observed for 12 minutes, and we recorded the plant of first choice, and the visitation time and number of flowers visited for each of the two plants. First choice was defined as the plant first contacted by the insect, with either a leaf or flower. If the pollinator did not make a choice within 5 minutes, it was recorded under 'no response' and the observation was terminated. Observations were performed using a handheld computer (Psion Workabout Pro™ 3, London, UK) programmed with The Observer XT software (version 10, Noldus Information Technology, Wageningen, The Netherlands). Each pollinator was used only once. Butterflies were 3-10 days old, starved circa 20 hours before the experiment and provided with a Brussels sprouts plant to lay eggs, and thus in this way we ensured that the observation time would be spent on feeding and not on oviposition. Syrphid flies were 5-15 days old, starved 4-8 hours before the experiment, and provided with a Brussels sprouts plant to lay eggs and some water to prevent dehydration. For each plant pair, 10-20 individuals per pollinator species were tested. If more than 10 individuals were non-responsive, observations for that day were terminated and data was excluded from the dataset. Experiments were carried out in a flight chamber set-up (gauze tent of 293 cm x 200 cm x 230 cm), in a greenhouse compartment (25 ± 1 °C, 50 -70 % r.h., L16:D8). For each plant treatment, 7-10 plant pairs were tested. After experimental use, we recorded the number of flowers and inflorescences for each plant.

Statistical analysis

For count data such as the number of flowers, inflorescences, and pollen grains we used generalized linear (mixed) models with a Poisson distribution and a log link function

or negative binomial distribution with a log link function to correct for overdispersion. Herbivore species was included in the model as fixed factor. For *post hoc* analysis we used Tukey's *post hoc* tests. Random factors were selected using a backwards approach; all random factors such as *block* (flowers and inflorescences), and *plant* (pollen grains), were initially added to the model and removed if they explained less than 5% of the variation or were statistically non-significant ($P > 0.05$). We used the lme4 (Bates et al. 2015), multcomp (Hothorn et al. 2008), and lmtest (Zeileis and Hothorn 2002) packages for these analyses. For continuous data such as total volatile emission, emission of compound classes or individual compounds, relative reflection of yellow and UV, amount of nectar, and average pollen size, we used linear (mixed) models with a Gaussian distribution and identity link function or a Gamma distribution with a log link function if the data did not follow a normal distribution. The same fixed factors, random factor selection approach, and packages as for count data were used. Random factor used was *plant* (yellow, UV and pollen size). For pollinator behaviour data (number of insects, flowers visited, time spent per plant, and flower) we used the proportion of the response variable between infested and uninfested plants. We used generalized linear mixed models with a Poisson distribution and a log link function. The response variable was fitted to the intercept, and random factor used was *plant pair*.

We analysed reflectance spectra with principal component analysis (PCA), PERMANOVA analyses, and support vector machines (SVMs) (Cortes and Vapnik 1995). Principal component analysis (PCA) was performed with MetaboAnalyst 4.0 with default settings (Xia et al. 2015). For PERMANOVA analyses, the difference in reflectance spectra between top and base parts of petals was analysed by including herbivore treatment, plant ID, flower ID, and petal part as fixed factors. The difference in reflectance spectra between herbivore treatments for top and base parts of petals were analysed by including herbivore treatment, flower ID, and plant ID as fixed factors. We used 999 permutations for all PERMANOVA analyses. We used the vegan package for these analyses (Oksanen et al. 2007). For SVM the reflectance spectra were divided in 64 features of 4-5 nm each. We trained the models with 60% of the data, used 20% for model optimization and cross-validation, and used 20% for testing. For each model, we tested a range of cost values ($2^{-2:7}$ with exponential steps of 1) and gamma values (from 0 to 1 with steps of 0.05) and selected the values which supported the best model. For gamma, values auto-selected by the model always supported the best models compared to a range of test values selected by us (between 0 and 1 with steps of 0.05), and consequently we used the values auto-selected by the model, which were always <0.01. Accuracy was assessed with 10-fold cross-validation. Error rate was calculated from the confusion matrix by dividing the sum of the diagonals by the sum of the total. The result was subtracted from 1. We used the e1071 package for these analysis (Meyer et al. 2017). For all reflectance spectra analyses we excluded wavelengths 421 to 498 from our dataset because of low

light intensity in this area of the deuterium-halogen light source.

We analysed volatile blend composition with cluster analysis. For cluster analysis, data were averaged per treatment, log transformed, and range scaled. Clustering was done using Euclidian distances as distance measure, and Ward's clustering criterion as clustering method. Bootstrapping was done with 1000 bootstrap replications. We used the pvclust package for these analysis (Suzuki and Shimodaira 2006). Heatmaps were produced with MetaboAnalyst 4.0 with default settings, except we did not use standardization (Xia et al. 2015).

We analysed pollen size distribution by comparing the pollen size distribution of uninfested and infested plants with a chi-square test. We used the fifer package for these analysis (Fife 2014). All analyses were carried out in R (version 3.4.3 × 64, 2017, The R Foundation for Statistical Computing Platform).

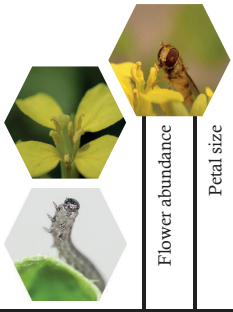
Results

Herbivore induction affected most flower traits and pollinator behaviour (Table 1). Changes in response to herbivory were observed for flower morphology, flower colour, the composition of the volatile blend, and nectar and pollen production of flowering *B. nigra.* Except for flower colour, specificity in induced traits and pollinator behaviour was herbivore species-specific and beyond classifications such as herbivore functional groups (HFG). For example, both pollinators had opposite responses to plants infested with the two aphid species (Table 1). Moreover, herbivore-induced changes were not restricted to specific herbivores or traits, and different herbivore species induced changes in various traits. Below, we provide details for the effects of plant responses to herbivory for each of the flower traits and the behaviour of the pollinators.

Effect of herbivore infestation on flower abundance, size and morphology

On average, plants had 364 flowers and 34 inflorescences one week after the start of flowering. Herbivory did not affect the number of inflorescences (Appendix C Fig. C1, GLMM: $\chi^2 = 2.20$, df = 5, $P = 0.821$), nor the number of flowers (Fig. C1, GLMM: $\chi^2 = 1.47$, df = 5, $P = 0.916$). Flowers had an average display area of 1.0 cm^2, and length and width of 1.2 by 1.0 cm. Herbivory affected the display size of flowers (Fig. C2, GLM: $\chi^2 = 46.3$, df = 5, $P < 0.001$), and flowers of plants infested with *P. xylostella* were 18% larger than flowers of uninfested plants (Tukey's *post hoc* tests, $P < 0.001$). Herbivory affected several shape characteristics of flowers (Fig. C2), such as major chord length (GLM: $\chi^2 = 36.5$, df = 5, $P < 0.001$), minor chord length (GLM: $\chi^2 = 25.3$, df = 5, $P < 0.001$), aspect ratio (GLM: $\chi^2 = 11.7$, df = 5, $P = 0.039$), solidity (GLM: $\chi^2 = 14.9$, df = 5, $P = 0.011$), and convexity (GLM: $\chi^2 = 12.7$, df = 5, $P = 0.026$). Herbivory did not affect

Table 1. Various flower traits of *Brassica nigra* and visitation by two pollinators for plants infested with different herbivores. Increase (+), decrease (-), or no effect (O) when compared with traits of uninfested plants. Letters for volatile blend and compound class are based on cluster analyses. Letters and numbers for reflectance spectrum are based on support vector machine models, where letters indicate differences between feeding guilds and numbers differences within feeding guilds. No entry indicates traits were not measured for the respective herbivore. Photographs show *Athalia rosae* larva (bottom), flower of *B. nigra* (centre), and adult syrphid fly *Episyrphus balteatus* feeding on pollen of *B. nigra* (top right). Photograph credits: Jitte Groothuis and Quint Rusman.



	Flower morphology						Flower colour			Plant volatiles			Flower rewards		Insect behaviour	
	Flower abundance	Petal size	Petal length	Petal width	Petal roundness	Display size	Reflectance spectrum	Yellow reflectance	UV reflectance	Total volatile emission	Volatile blend	Benzenoids and phenylpropanoids	Nectar production	Pollen production	Syrphid fly behaviour	Butterfly behaviour
Athalia rosae	O	O	_	O	_	O	A1	_	O	O	A	B	_		O	O
Plutella xylostella	O	O	O	+	_	+	A2	O	O	O	C	A			O	_
Pieris brassicae													_	O		
Brevicoryne brassicae	O	O	_	O	_	O	B1	O	+	O	A	A	_		_	_
Lipaphis erysimi	O	+	O	+	_	O	B2	O	+	O	B	C	O	O	+	+
Delia radicum	O	+	O	+	_	O	C	O	O	O	C	C	O	_	+	O

flower eccentricity (LM: $\chi^2 = 5.4$, df = 5, $P = 0.368$). Petals had an average surface area of 0.13 cm^2, and length and width of 0.5 by 0.3 cm. Herbivory affected the surface area of petals (Fig. C3, GLM: $\chi^2 = 40.9$, df = 5, $P < 0.001$), and petals of plants infested with *L. erysimi* or *D. radicum* were 7% larger than uninfested plants (Tukey's *post hoc* tests, $P = 0.008$, $P = 0.010$ respectively). Herbivory affected several shape characteristics of petals (Fig. C3), such as major chord length (GLM: $\chi^2 = 79.0$, df = 5, $P < 0.001$), minor chord length (GLM: $\chi^2 = 37.3$, df = 5, $P < 0.001$), aspect ratio (GLM: $\chi^2 = 52.3$ df = 5, $P < 0.001$), and eccentricity (GLM: $\chi^2 = 59.0$, df = 5, $P < 0.001$). Overall, petals of herbivore-infested plants had smaller aspect ratios and eccentricity than uninfested plants, caused by shorter (*A. rosae* and *B. brassicae*) or broader (*P. xylostella*, *L. erysimi*, *D. radicum*) petals. Despite changes in petal size caused by all herbivores, only flowers of plants infested with *P. xylostella* had larger display size and smaller solidity than uninfested plants.

Effect of herbivore infestation on flower colour

The reflectance spectra of the top and base part of petals differed significantly (Fig. C4, PERMANOVA: R2 = 67.1, df = 1, $P < 0.001$). The reflectance spectra of the top part of petals included 2 maximum reflectance peaks at 326 and 351 nm compared with the reflectance spectra of the base part of petals. Because of these differences, we performed separate analyses for the effects of herbivory for the top and base part of petals. The colour of both top and base parts of petals of *B. nigra* was affected by herbivory (Fig. C5, Top: PERMANOVA: R2 = 7.8, df = 5, $P < 0.001$; Base: PERMANOVA: R =9.7, df =5, $P < 0.001$). The SVM models were accurate in identifying herbivore treatments of individual plants as defined by the training datasets, based on the reflectance spectra of both top and base parts of petals (Table C2 + C3; Top: SVM accuracy 89%, error rate 10%, Base: SVM accuracy 83%, error rate 12%). This indicates herbivore-species-specific changes in the reflectance spectra of both top and base parts of petals, and thus significant differences between all treatments. In addition, the SVM models were accurate in identifying HFG treatments of individual plants as defined by the training datasets (Tables C2 and C3; Top: SVM accuracy 98%, error rate 2%, Base: SVM accuracy 92%, error rate 4%). This indicates HFG-specific changes, and thus significant differences between herbivores of different HFGs. This was confirmed by the ability of SVM models to assign plants infested with individual herbivore species to the correct HFGs and *vice versa* (Table C2 and C3).

Spectral profiles of petals of *B. nigra* contained two regions of interest; the yellow/orange region (570-650 nm), and the UV region (310-370 nm). The relative diffuse reflectance of yellow/orange of the top part of petals was affected by herbivory (Fig. 1, GLM: $\chi^2 = 28.48$, df = 5, $P < 0.001$), as well as the base part of petals (Fig. 1, GLM: $\chi^2 = 27.634$, df = 5, $P < 0.001$). Petals of plants infested with *A. rosae* reflected 5-9 % less yellow/orange than all other treatments except plants infested with *B. brassicae* (Fig. 1). The relative diffuse reflectance of UV of the top part of petals was affected by herbivory (Fig. 1, GLM: $\chi^2 = 29.66$, df = 5, $P < 0.001$). Flowers of plants infested with *B. brassicae* and *L. erysimi* reflected 8-13 % more UV than uninfested plants, and this resulted in a reduced yellow/UV ratio (Fig. 1).

Effect of herbivore infestation on plant volatile emission

The volatile profile of flowering *B. nigra* plants consisted of 51 compounds of 6 major classes: benzenoids and phenylpropanoids, monoterpenoids, homoterpenoids, sesquiterpenoids, fatty-acid and amino-acid derivatives, and nitrogen containing compounds (see Table C4). Herbivore infestation did not affect total volatile emission (Fig. C6, LM: $\chi^2 = 4.84$, df = 5, $P = 0.436$) and did not result in qualitative differences

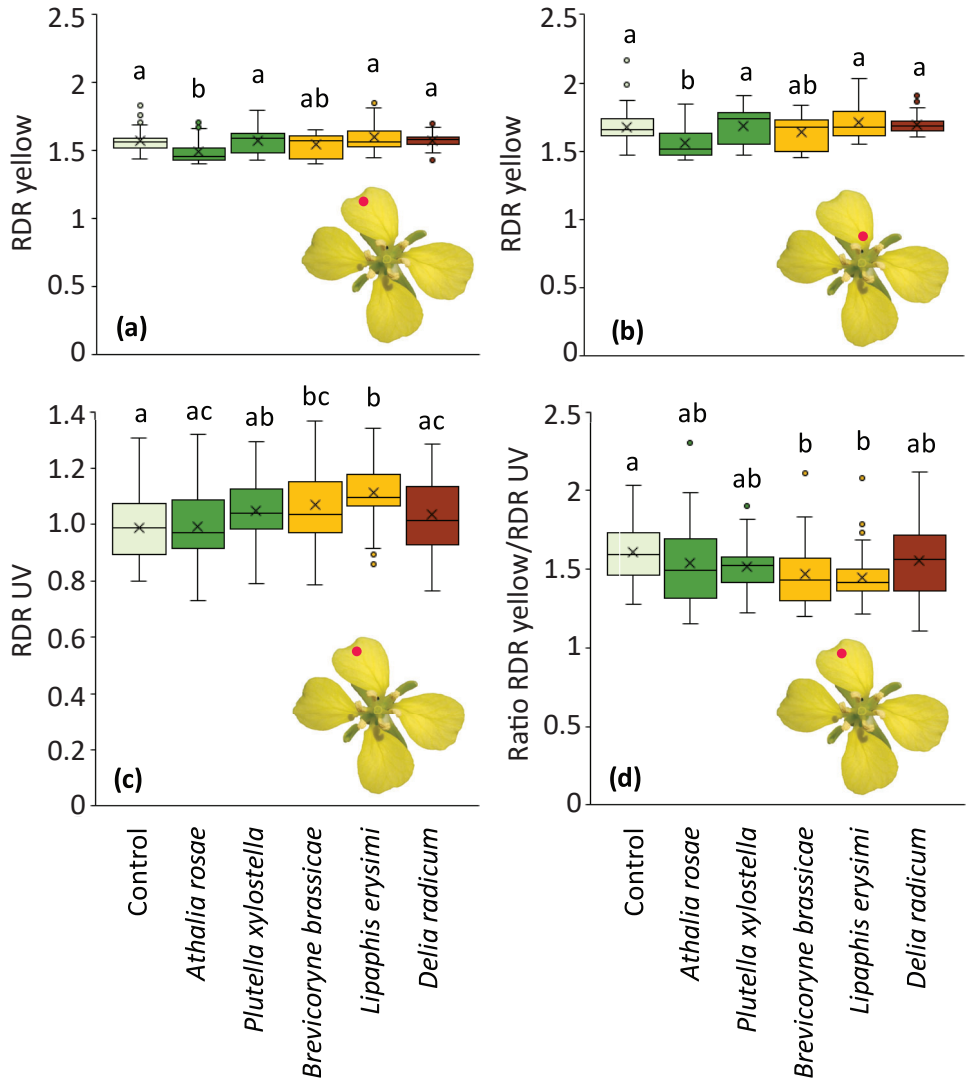


Fig. 1. Relative diffuse reflection (RDR) of yellow (570-650 nm) and UV (310-370 nm) wavelengths by petals of uninfested *Brassica nigra* plants or plants infested with different herbivores. (a) Relative diffuse reflection of yellow of top parts of petals. (b) Relative diffuse reflection of yellow of base parts of petals. (c) Relative diffuse reflection of UV of top parts of petals. (d) Ratio RDR yellow/ RDR UV of top parts of petals. Boxplots show median (line), mean (x), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. The red dot on the flower images indicate where measurements were taken (top or base), which was done after seven days of herbivory. Number of replicates per herbivore treatment varied between six and eight plants. From each plant, six flowers were used, of which each petal was measured, both top and base parts. Letters above bars indicate significant differences at $\alpha = 0.05$ based on Tukey's *post hoc* tests.

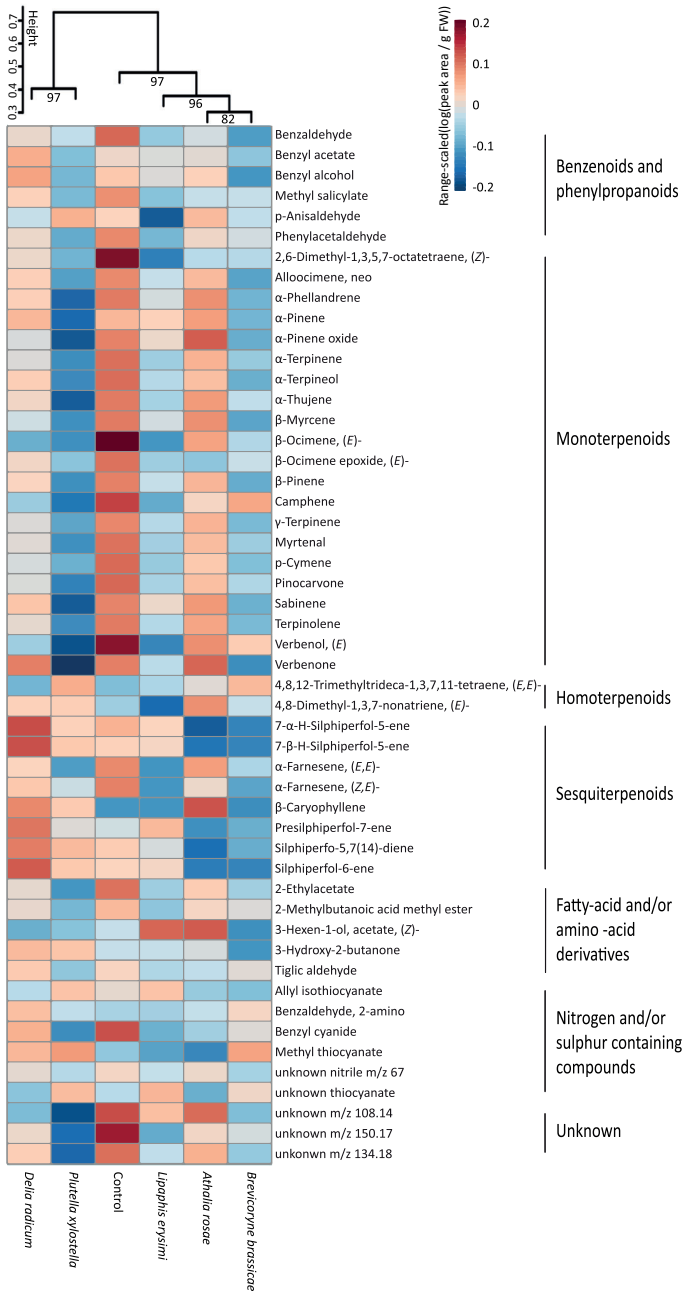


Fig. 2. Dendrogram and heat map of the emission of volatile compounds of *Brassica nigra* plants infested with different herbivores or uninfested plants. Dendrogram clustering was performed using Ward's clustering algorithm with Euclidean distances. Values in the dendrogram are approximately unbiased probability values, where values ≥ 95 indicate significant differences. For the heat map, we used range-scaled log transformed values of volatile emission (peak area / g FW) for each compound. Volatiles were collected after seven days of herbivory. Number of replicates per herbivore treatment varied between seven and nine plants.

in the volatile profiles compared to uninfested plants. Herbivore infestation resulted in quantitative differences in the volatile profiles of infested and uninfested plants (Fig. 2). The largest difference was between plants infested with caterpillars of *P. xylostella* and the root herbivore *D. radicum*, and all other treatments. The volatile profile of uninfested plants differed from that of infested plants, where infested plants emitted lower amounts of benzenoids and phenylpropanoids (LM: $\chi^2 = 5.85$, df = 1, $P = 0.016$), and tended to emit lower amounts of monoterpenoids (LM: $\chi^2 = 3.25$, df = 1, $P = 0.071$). All herbivores differently affected the volatile profile by changing the emission of specific compounds, whereas the volatile profile of plants infested with *A. rosae* and *B. brassicae* and plants infested with *P. xylostella* and *D. radicum* wererelatively similar (Fig. 2). Moreover, herbivores differently affected volatile compound classes, which is evident from different clustering of the herbivores for each volatile compound class (Fig. C7). Interestingly, herbivore-induced changes in benzenoids and phenylpropanoids seem to match the observed herbivore-induced changes in pollinator behaviour quite well (Table 1). We did not observe distinct clustering of volatile profiles based on herbivore feeding guild or site. Thus, herbivore-induced plant volatile profiles were specific for each attacking herbivore species within guild or feeding site.

Effect of herbivore infestation on flower rewards

Plants produced on average 0.26 µl nectar and 47,768 pollen grains per flower. Nectar production of flowers was affected by herbivory (Fig. 3, LM: F=6.61, df = 5, $P < 0.001$). Plants infested with *A. rosae* or *B. brassicae* produced less nectar than uninfested plants (Tukey's *post hoc* tests, $P = 0.037$ and $P = 0.013$ respectively), plants infested with *L. erysimi* (Tukey's *post hoc* tests, $P = 0.002$ and $P < 0.001$ respectively), or *D. radicum* (Tukey's *post hoc* tests, $P = 0.072$ and $P = 0.029$ respectively). The number of pollen grains produced by flowers was affected by herbivory (Fig. 3, GLM: $\chi^2 = 10.09$, df = 3, $P = 0.018$). Plants infested with *D. radicum* produced fewer pollen grains than uninfested plants (Tukey's *post hoc* tests, $P = 0.029$), and plants infested with *P. brassicae* (Tukey's *post hoc* tests, $P = 0.024$). The average size of pollen grains did not differ between uninfested or herbivore-infested plants (Fig. C8, GLM: $\chi^2 = 0.18$, df = 3, $P = 0.981$), nor did the pollen size distribution (Fig. C9, chi-square test, $P = 1.000$).

Effect of herbivore infestation on pollinator behaviour

We observed the behaviour of 902 responsive pollinators, with 108.4 h of observation time, and over 8,000 flower visits and these observations revealed that the behaviour of both butterflies and syrphid flies was affected by herbivore infestation. The direction of the effect depended on herbivore and pollinator species. Butterflies landed less frequently

on plants infested with *P. xylostella* (GLMM: z = -2.25, *P* = 0.025) or *B. brassicae* (GLMM: z = -3.29, *P* = 0.001), and more frequently plants infested with *L. erysimi* (GLMM: z = 3.13, *P* = 0.002) than on uninfested plants (Fig. 4). Butterflies landed as frequently on plants infested with *A. rosae* (GLMM: z = -0.37, *P* = 0.715) or *D. radicum* (GLMM: z = 0.23, *P* = 0.816) as they did on uninfested plants. Herbivore infestation had the same effect on the duration of visitation and the number of flowers visited as on the landing preference for butterflies (Fig. 4). Syrphid flies landed more frequently on plants infested with *L. erysimi* (GLMM: z = 2.09, *P* = 0.036) or *D. radicum* (GLMM: z = 2.23, *P* = 0.026), and tended to land less frequently on plants infested with *B. brassicae* (GLMM: z = -1.76, *P* = 0.079) than on uninfested plants (Fig. C10). Syrphid flies landed as frequently on plants infested with *A. rosae* (GLMM: z = -0.62, *P* = 0.537) or *P. xylostella* (GLMM: z = -0.59, *P* = 0.557) as they did on uninfested plants. Herbivore infestation had the same effect on the duration of visitation and the number of flowers visited as on the landing preference for syrphid flies (Fig. C10).

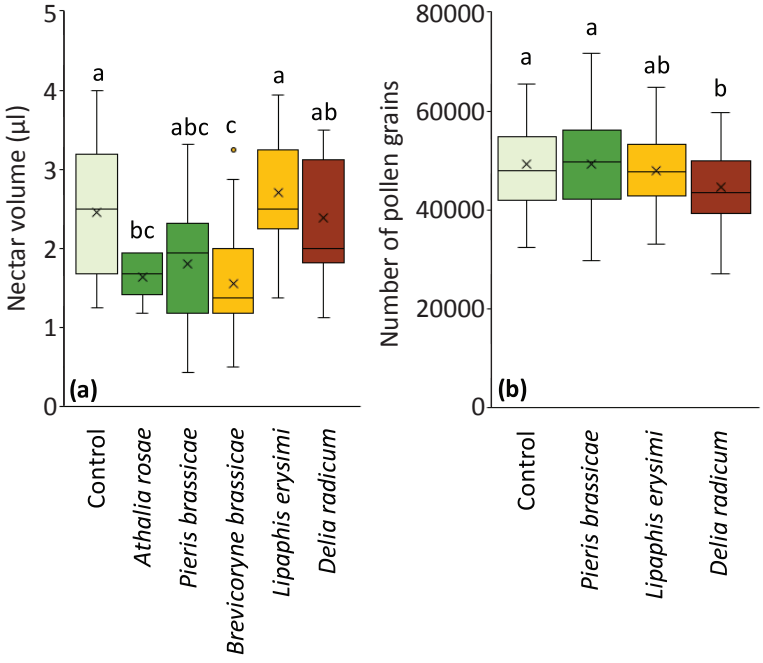


Fig. 3. Nectar volume and number of pollen grains of uninfested *Brassica nigra* plants or plants infested with different herbivores. a) Nectar volume of eight flowers of uninfested *B. nigra* plants or plants infested with different herbivores. Number of replicates per herbivore treatment varied between 14 and 15 plants. b) Number of pollen grains of one flower of uninfested *B. nigra* plants or plants infested with different herbivores. Boxplots show median (line), mean (x), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Nectar volume and number of pollen grains were measured after seven days of herbivory. Number of replicates per herbivore treatment was 10 plants, per plant we measured pollen for five flowers. Letters above bars indicate significant differences at $\alpha = 0.05$ based on Tukey's *post hoc* tests.

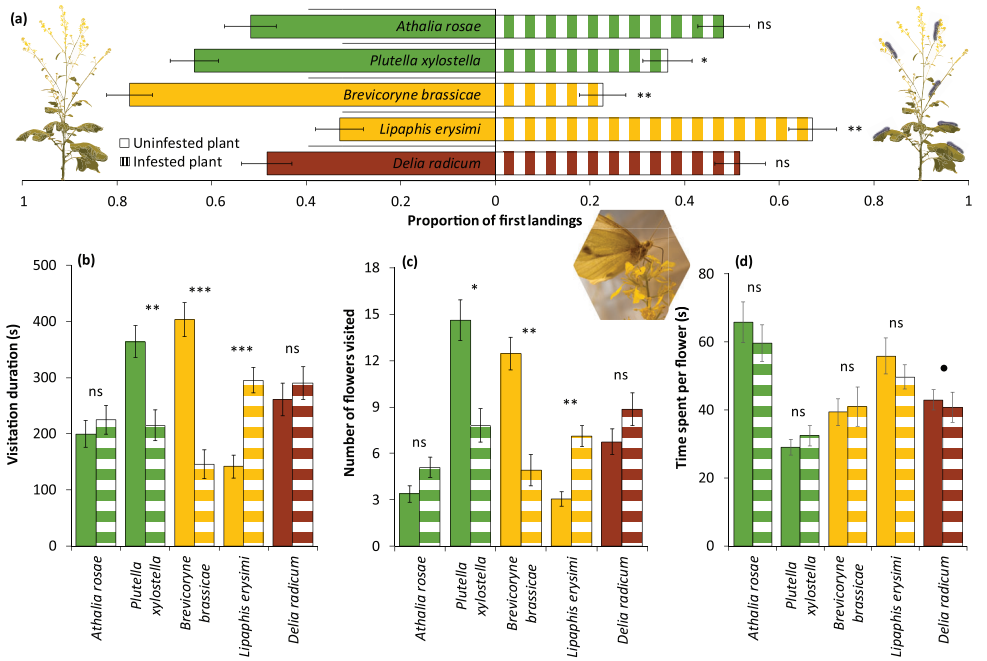


Fig. 4. Preferences of the butterfly *Pieris brassicae* for uninfested *Brassica nigra* plants or plants infested with different herbivores. a) Proportion of *P. brassicae* butterflies (mean ± SE) that first landed on flowers or leaves of *B. nigra* plants infested with different herbivores or uninfested plants. b) Visitation duration (mean ± SE); c) number of flowers visited (mean ± SE); and d) time spent per flower (mean ± SE) by individual pollinators on infested or uninfested *B. nigra* plants. Butterfly behaviour was assessed after seven days of herbivory. Number of replicates per herbivore treatment varied between 75 and 89 butterflies, and 8 and 10 plant pairs. Asterisks above bars indicate significant differences with *** = $P < 0.001$, ** = $0.001 \geq P < 0.01$, * = $0.01 \geq P \leq 0.05$, and • = $0.05 > P < 0.1$, based on Tukey's *post hoc* tests. Photograph shows a *P. brassicae* butterfly visiting flowers of *B. nigra*. Photograph credit: Quint Rusman.

Discussion

Our data show that responses of *B. nigra* to attack by different herbivores changed multiple flower traits and affected pollinator behaviour. Flower traits that most strongly changed in response to herbivory were flower morphology, flower colour, the composition of the volatile blend, and nectar production. For most traits, specificity is beyond feeding guild and site, and changes are herbivore-species-specific. The herbivore-induced species-specific changes in flower traits resulted in herbivore-induced species-specific changes in pollinator behaviour. The best predictors for the changes in pollinator behaviour were the combined changes in all flower traits, or changes in specific compounds within the volatile blend, especially benzenoids and phenylpropanoids. Changes in flower morphology and nectar production could explain pollinator behaviour in some cases, but not in others. Thus, flowers show extensive plasticity in response to antagonistic

herbivores, with contrasting effects on mutualistic pollinators.

Flowering plants appear to be highly plastic in response to herbivory. Specificity in herbivore-induced changes in flower traits yielded a unique phenotype to each herbivore attacker, with species-specific effects on different pollinators. Our data does not support the predicted specificity of elicitation or effect based on herbivore feeding guild and site (Rusman et al. 2018 - chapter 3). This prediction was based on specificity of effect of pollinator functional groups to herbivory (Rusman et al. 2018 - chapter 3), and specificity of elicitation of phytohormones involved in defence regulation (JA and SA) by herbivores from different feeding guilds and that use different feeding sites (Ali and Agrawal 2012, Thaler et al. 2012, Johnson et al. 2016). Within pollinator functional groups, pollinator species can respond differently to plants exposed to the same herbivore (Hoffmeister et al. 2016). Apparently, specific effects on pollinator functional groups do not accurately predict specificity of elicitation of herbivore-induced changes in flower traits. Moreover, specificity in elicitation of the phytohormones JA and SA may not accurately predict the induced phenotype, because of alternative signalling pathways and spatiotemporal modulators of the JA response (Erb et al. 2012). Despite effects of herbivore feeding guild on phytohormone induction and related gene transcription (De Vos et al. 2005, Bidart-Bouzat and Kliebenstein 2011), the resulting induced phenotype is at least partially herbivore species-specific (Heidel and Baldwin 2004, Travers-Martin and Müller 2007, Bidart-Bouzat and Kliebenstein 2011, Chung and Felton 2011). The same seems to be true for the floral phenotype. For example, plant responses to root- and leaf-chewing herbivores, which are both mediated by JA, had differential effects on flower traits such as petal size (Poveda et al. 2005), flower abundance (Barber et al. 2011), and flowering phenology (Poveda et al. 2003). Application of JA reduced nectar production in *B. nigra*, whereas herbivory by *P. rapae* or *P. brassicae* caterpillars, which mainly induce JA, increased or had no effect on nectar production (Bruinsma et al. 2008, Bruinsma et al. 2014). Herbivory by two aphid species had differential effects on floral volatile emission (Pareja et al. 2012). Thus, specificity of herbivore-induced floral traits is herbivore-species-specific, and results in specificity of effects on pollinator visitation.

Herbivores induce changes in multiple components of the flower phenotype, which makes it difficult to disentangle the contribution of each component to the changes in pollinator behaviour. Plasticity in any traits might contribute to altered pollinator visitation. Moreover, because plants use multiple flower traits to attract pollinators (Leonard et al. 2012, Junker and Parachnowitsch 2015), the combination of all changes in flower traits might explain pollinator visitation best. Herbivore-induced changes in individual flower traits might shift the relative importance of each flower trait within the complete floral phenotype for pollinator attraction (Leonard et al. 2012, Lawson et al. 2017). Still, some flower traits seem to be more plastic than others and might contribute more to pollinator attraction, more so when the trait provides information

on floral rewards. Especially floral volatiles seem to undergo profound changes in response to herbivory (Kessler and Halitschke 2009, Pareja et al. 2012, Schiestl et al. 2014, Cozzolino et al. 2015, Lucas-Barbosa et al. 2016), and may explain changes in pollinator behaviour well (Kessler and Halitschke 2009, Schiestl et al. 2014), although it is often unclear which individual compounds are used by pollinators (but see Knauer and Schiestl (2015)). Other flower traits like colour and morphology also change in response to herbivory and are important for pollinator attraction (Strauss et al. 1996, Campbell et al. 2010, Hempel de Ibarra et al. 2015). Flower volatiles, colour, and morphology can provide information on floral rewards for naïve pollinators (Raine and Chittka 2007, Gómez et al. 2008, Haverkamp et al. 2016), conferring so called "honest signals", which would predict adaptive responses of naïve pollinators to herbivore-induced changes in flower volatiles, colour or morphology. However, herbivory also resulted in changes in floral rewards, which could potentially disrupt honest signalling. In this study, the combined changes in all flower traits, or the induced changes in specific plant volatile compounds, especially in benzenoids and phenylpropanoids, seem to predict herbivore-species-specific changes in pollinator behaviour best. Changes in flower morphology and nectar production could predict pollinator behaviour in some cases, but not in others. We did not investigate nectar and pollen composition, which may change in response to herbivory (Adler et al. 2006, Bruinsma et al. 2014), but do not always predict pollinator behaviour well (Vanderplanck et al. 2014, Carr et al. 2015). Although the mere presence of the herbivores on the plants could have affected pollinator behaviour directly, we never observed that pollinators would be especially attracted or repelled by herbivore-infested inflorescences or the herbivores themselves in the greenhouse or field (pers. obs. Quint Rusman & Dani Lucas-Barbosa). To identify the exact cause of herbivore-induced changes on pollinator behaviour, future studies should manipulate individual flower traits and their combinations or use natural variation in flower traits to assess which traits most strongly affect flower visitation by different pollinator species. This will promote understanding of why flowers show such extensive plasticity to herbivore attack.

Here, our data show that responses of *B. nigra* plants to herbivores that regularly attack this plant species can have positive, negative or neutral effects on pollinator behaviour, and that different pollinators respond differently to herbivore-induced changes in flower traits. This is supported by evidence from field studies, where multiple herbivores have been shown to differentially affect pollinator community composition, with positive, negative or neutral effects on the visitation by pollinator species (Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3). Changes in the behaviour of one or more pollinator groups and resulting changes in plant reproduction might confer considerable ecological costs of herbivore-induced changes in flower traits. Interestingly, some plant species seem to be able to maintain overall pollinator visitation in the field (Barber et al. 2011,

Lucas-Barbosa et al. 2013, Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3). Thus, despite the repellence of some pollinators by herbivore-induced changes in flower traits, plants seem to compensate by attracting other pollinators. In this way, defences can be activated without compromising reproduction (Lucas-Barbosa et al. 2013, Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3), even when defence and flower traits are tightly linked (Lucas-Barbosa 2016, Jacobsen and Raguso 2018). Alternatively, herbivore-induced changes in flower traits as by-products of the plant's inducible defensive responses may be maintained because they do not affect reproduction (Gould and Lewontin 1979). Thus, ecological costs of flower plasticity in response to herbivory *via* disruptions of plant-pollinator interactions might be limited considering the entire pollinator community and, in particular for generalized pollinator systems.

Our study shows that plant responses to herbivores feeding on the flowers, leaves or roots have profound effects on the flower phenotype and pollinator visitation. Plant responses to any herbivore changed two or more traits, including flower morphological, chemical (colours and volatiles), and reward traits, and changes were herbivore-species-specific. This highlights the importance of exploring plant responses to multiple herbivores and pollinators and measuring multiple flower traits to reveal the underlying mechanisms of plant-mediated herbivore-pollinator interactions. Herbivores potentially play a significant role as agents of selection on floral traits and plant reproduction *via* plant-mediated interactions with pollinators.

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Chapter 6

Settling on leaves or flowers: Herbivore feeding site determines the outcome of indirect interactions between herbivores and pollinators

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Submitted

Abstract

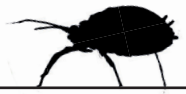
For antagonist and mutualist community members, indirect interactions can impact community structure and trait evolution, influencing eco-evolutionary dynamics. In nature, antagonist and mutualist networks are intertwined, but how trait variation in herbivore species affects plant interactions with mutualists has seldom been explored. We investigated the effect of trait variation in feeding behavior of plant antagonists (herbivorous insects) on the visitation by plant mutualists (pollinators) on flowering *Brassica nigra* plants. We placed herbivores on either leaves or flowers, and recorded the responses of two pollinator species when visiting flowers. Our results show that trait variation in antagonist feeding behavior has profound impact on the outcome of antagonist-mutualist interactions. Herbivores feeding on flowers had consistent positive effects on pollinator visitation, whereas herbivores feeding on leaves did not. Herbivores themselves preferred to feed on flowers, and mostly performed best on flowers. We conclude that feeding site choice in antagonists can profoundly affect antagonist-mutualist interactions and thereby show the potential for eco-evolutionary dynamics between antagonists and mutualists.

Keywords: Antagonist-mutualist interactions, eco-evolutionary dynamics, herbivore-induced plant responses, flower visitors, plant defence, plant-mediated interactions, preference-performance, trait variation

Introduction

Ecological communities are structured by direct and indirect interactions. Direct interactions result from the physical interaction between two species, whereas indirect interactions are established through an intermediary species (Wootton 1994). Most communities consist of more than two species, in which we typically observe indirect interactions between species. Indirect interactions not only play important roles in natural systems by having considerable impact on community organization and ecosystem processes (Werner and Peacor 2003, Schmitz et al. 2010), but also by promoting evolutionary change and thereby driving trait distributions in communities (Strauss et al. 2005, Turcotte et al. 2012, Guimarães Jr et al. 2017). Effects of indirect interactions on community organization and trait evolution generate eco-evolutionary feedback because indirect interactions are often trait-mediated, and trait evolution subsequently shapes indirect interactions (Walsh 2013). Thus, indirect interactions are highly integrated in ecological and evolutionary dynamics of multispecies communities.

Indirect interactions can drive eco-evolutionary dynamics in antagonist and mutualist networks. Most research has focused on indirect antagonistic interactions, where trait variation in feeding behavior can affect other community members *via* indirect interactions (Johnson et al. 2009, Farkas et al. 2013, Matthews et al. 2016, Ohgushi 2016), and changes in the community in turn affect trait evolution by changing selection regimes (Siepielski and Benkman 2004, Agrawal et al. 2012, Bassar et al. 2012). Likewise, indirect interactions in mutualist networks can affect network structure (Cahill et al. 2008, Rodríguez-Echeverría and Traveset 2015, Rodríguez-Echeverría et al. 2016), and shape trait evolution (Guimarães Jr et al. 2011, Guimarães Jr et al. 2017), which in turn shifts interaction networks (Santamaría and Rodríguez-Gironés 2007, Nuismer et al. 2013). Antagonistic and mutualistic community members have mostly been investigated in isolation, as separate networks, despite most (if not all) natural communities containing both antagonists and mutualists (Fontaine et al. 2011). Modelling exercises suggest that antagonists can profoundly affect mutualistic community members and evolutionary dynamics of mutualist networks (Nuismer et al. 1999, Bronstein et al. 2003). Indeed, field collected evidences of indirect effects on community dynamics have been accumulating (Steets et al. 2006, Gehring and Bennett 2009, Ghyselen et al. 2016, Hoffmeister et al. 2016, Chautá et al. 2017, Rusman et al. 2018 - chapter 3). In addition, plant antagonists can alter selection on plant traits *via* indirect interactions with plant mutualists (Ashman 2002, Irwin 2006). Still, eco-evolutionary dynamics have rarely been explored for antagonist-mutualist interactions (Fontaine et al. 2011), and we have limited knowledge of how antagonist-mutualist interactions affect community organization and trait evolution. To gain insight in this, a first crucial step is to investigate how trait variation in antagonists affects antagonist-mutualist interactions.



Flowering plants are frequently exposed to antagonist-mutualist interactions because flowering plants are attacked by herbivores (antagonists) and at the same time interact with pollinators (mutualists) for reproduction. Plants use a variety of flower traits to attract pollinators (Junker and Parachnowitsch 2015), and these traits readily change upon herbivore attack (Lucas-Barbosa 2016, Rusman et al. 2019 - chapter 5). As a consequence, the attraction and visitation of pollinators can be negatively affected, but positive and neutral effects have also been found (Kessler and Halitschke 2009, Lucas-Barbosa 2016, Rusman et al. 2019 - chapter 5). Furthermore, flowering plants are attacked by herbivores feeding on roots, leaves and flowers. Some herbivores are obligate folivores or florivores, whereas others move from leaves to flowers at some point in time (Agerbirk et al. 2010, Bandeili and Müller 2010, Lucas-Barbosa et al. 2013). Trait variation in herbivore feeding behavior affects plant responses to herbivory (Ali and Agrawal 2012), and is important for the outcome of indirect interactions among plant antagonists (Utsumi and Shefferson 2015). It has been hypothesized that trait variation in herbivore feeding behavior can determine the outcome of herbivore-pollinator (antagonist-mutualist) interactions (Kessler and Halitschke 2009), and subsequently alter the composition of mutualist networks (Rusman et al. 2018 - chapter 3). Moreover, feeding behavior of the herbivore might be key in determining how plants deal with the potential trade-off between reproduction and defense mechanisms (Kessler and Halitschke 2009, Lucas-Barbosa 2016). Herbivores feeding on different feeding sites require different plant defense strategies, with varying direct and indirect effects on plant reproduction.

In this study, we tested whether antagonistic herbivores feeding on leaves or flowers of Black mustard affect interactions with mutualistic pollinators. We specifically studied (i) how the behavior of two pollinator species, the butterfly *Pieris brassicae* and the syrphid fly *Episyrphus balteatus,* were affected by plant exposure to different herbivores, (ii) if herbivore-induced changes in pollinator behavior were determined by feeding site of the herbivores on leaves or flowers, (iii) if the three herbivore species – the aphids *Brevicoryne brassicae, Lipaphis erysimi*, and *Myzus persicae* – had a preference for leaves or flowers, and (iv) how feeding site affected the performance of the herbivores themselves. We show that the outcome of indirect plant-mediated interactions between antagonists and mutualists is largely determined by the feeding site of the antagonist: herbivores feeding on flowers had a consistent positive effect on the attraction of different pollinators, whereas herbivores feeding on leaves did not. The evolution of antagonist feeding behavior might thus be important for mutualist network assembly *via* trait-mediated interactions.

Materials and Methods

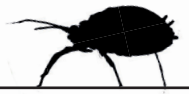
Plant and insects

Black mustard (*Brassica nigra*) seeds (accession CGN06619) were obtained from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands) and propagated by open-pollination in the field. Seeds were germinated in trays and one-week-old plants were transplanted and cultivated in pots (∅ 17 cm – 2 L) filled with potting soil (Lentse potgrond) and sand in a 1:1 volume ratio under greenhouse conditions (23 ± 2 °C, 50 -70 % r.h., L16:D8). Plants were used in the experiments once they started flowering (5/6 weeks old).

We used three aphid species for the experiments: *Brevicoryne brassicae*, *Lipaphis erysimi*, and *Myzus persicae*. In nature, all three aphid species are regularly found on both leaves and flowers of *B. nigra* (pers. obs. Quint Rusman, Lucille Chrétien, Daan Mertens). Aphids were originally collected in the surroundings of Wageningen (The Netherlands), and are routinely reared in the Laboratory of Entomology (Wageningen University) under greenhouse conditions (22 ± 1 °C, 50 -70 % r.h., L16:D8). *Brevicoryne brassicae* was reared on Brussels sprouts plants (*Brassica oleracea* variety *gemmifera* cultivar Cyrus); *L. erysimi* and *M. persicae* were reared on *Raphanus sativus*. We used two pollinator species for our experiments: The butterfly *Pieris brassicae* and syrphid fly *Episyrphus balteatus*. *Episyrphus balteatus* is a common flower visitor and efficient pollinator of Brassicaceae (Jauker and Wolters 2008), whereas *P. brassicae* has a low visitation frequency on *B. nigra* compared to other pollinators in the field (Lucas-Barbosa et al. 2013, Rusman et al. 2018 - chapter 3), but may nonetheless be important for long-distance pollen dispersal (Courtney et al. 1982). *Pieris brassicae* are routinely reared at the Laboratory of Entomology (Wageningen University) under greenhouse conditions (22 ± 1 °C, 50 -70 % r.h., L16:D8). Larvae were reared on Brussels sprouts plants (*Brassica oleracea* variety *gemmifera* cultivar Cyrus) and adult butterflies were given a honey solution (10%). *Episyrphus balteatus* were obtained as pupae from Katz Biotech AG, Barutch, Germany. Once adult syrphid flies eclosed they were provided with sugar, pollen, water and a Brussels sprouts plant infested with *B. brassicae* aphids until the experiment.

Effect of herbivore feeding site on pollinator behavior

To investigate if pollinator behavior was influenced by the feeding site of the herbivore, we recorded the behavior of two pollinator species, the butterfly *P. brassicae*, and the syrphid fly *E. balteatus*, in two-choice situations. Individual pollinators were offered a choice between an uninfested plant and a plant infested with one of the herbivores, on either leaves or flowers (see below). A single butterfly or syrphid fly was released at a time, and at 100 cm from the plants. Each individual insect was observed for 12 minutes.



We recorded first choice for one of the two plants, the duration of the visitation, and number of flowers visited for each of the two plants. First choice was defined as the plant the insect had first contact with, either with a leaf or flower. If the pollinator did not make a choice within 5 minutes, it was recorded as 'no response', and the observation ended. Observations were performed using a handheld computer (Psion Workabout Pro^{tm} 3, London, UK) programmed with The Observer XT software (version 10, Noldus Information Technology, Wageningen, The Netherlands). Each insect was used only once. Butterflies were used for the experiments at 2-3 days after mating and 3-10 days since eclosion from pupae. They were starved for about 20 h prior to the bioassay and provided with a Brussels sprouts plant to lay eggs so that the observed behavior was driven by searching for food, and not for oviposition sites. Syrphid flies were 5-15 days old since eclosion, starved for 4-8 hours before the experiment, and provided with a Brussels sprouts plant infested with *B. brassicae* to lay eggs and some water to prevent dehydration. For each plant pair, 10-20 insects were tested. If more than 10 insects were non-responsive, observations were terminated that day. For each plant treatment, 10-11 plant pairs were tested. Experiments were carried out in a flight chamber set-up (gauze tent of 293 cm x 200 cm x 230 cm), in a greenhouse compartment (25 ± 1 °C, 50 - 70 % r.h., L16:D8), from the end of September (2016) till the beginning of March (2017).

Herbivore preference for leaves or flowers

To investigate if aphids have a preference for vegetative or flowering plant tissues, we recorded feeding site chosen by winged aphids on *B. nigra* plants. A flowering plant was placed in a mesh tent (95 x 95 x 190 cm) where 20 winged aphids of one of the three species were released – *B. brassicae*, *L. erysimi*, or *M. persicae.* The winged aphids were placed in a Petri dish (diameter 9 cm) on top of a wooden pedestal (height 38 cm); this pedestal stood at approximately 50 cm from the flowering plant. Aphids had 24 h to make a choice between vegetative (young leaves, old leaves and stems) and inflorescence tissues (buds, flowers, bracts and floral stems). Aphids recorded elsewhere in the tent than on the plant were considered unresponsive. Experiments were carried out in a greenhouse compartment (23 ± 1 ° C, 50 - 70 % r.h., L16:D8) from the beginning of October (2016) till the beginning of November (2016), and for each aphid species, feeding site preference was tested for 15 plants.

Herbivore performance on leaves or flowers

To investigate on which tissues aphids perform best, flowering *B. nigra* plants were infested with *B. brassicae*, *L. erysimi*, or *M. persicae*, on either leaves or flowers. We placed 20 adult female aphids on either two true leaves, 10 per leaf, or on four inflorescences, 5 per

inflorescence, on the final inflorescences of the four top flowering branches. To prevent aphids from moving between vegetative and flowering parts of the plant, we attached cotton wool with a small piece of a wire around the main stem between the vegetative and flowering part of the plant. The number of aphids was recorded 7 days after infestation as a proxy of performance. For the first six plants, the number of aphids was both counted and estimated. Since estimations closely matched counting while significantly reducing counting time, only estimations were used to assess the total number of aphids per plant. Experiments were carried out in a greenhouse compartment (23 ± 1 °C, 50 - 70 % r.h., L16:D8) from the end of September (2016) till the end of February (2017), and we had 25 to 28 plants per treatment. After assessing aphid performance, plants were used in the pollinator behavior experiment.

Statistical analysis

For pollinator behavior data (number of insects, flowers visited, time spent per plant, and flower) we used the proportion of the response variable between infested and uninfested plants. We used generalized linear mixed models with a Poisson distribution and a log link function. The response variable was fitted to the intercept, and *plant pair* was used as a random factor. For aphid preference and performance, we used generalized linear models with a Poisson distribution and a log link function or negative binomial distribution with a log link function to correct for overdispersion. Herbivore species, feeding site, and the interaction between herbivore species and feeding site were included in the model as fixed factors. Interactions were removed from the model if they were statistically non-significant ($P > 0.05$). For *post hoc* analysis we used Tukey's *post hoc* tests. For aphid preference, we corrected for the number of unresponsive aphids by including total number of aphids (responsive + unresponsive) as an offset. We used the lme4 (Bates et al. 2015), multcomp (Hothorn et al. 2008), and lmtest (Zeileis and Hothorn 2002) packages for these analyses. For correlations between numbers of aphids and visitation parameters of pollinators, we computed the correlation coefficient using the Pearson or Kendall method, depending on the distribution of the data. All analyses were carried out in R (version 3.4.3 × 64, 2017, The R Foundation for Statistical Computing Platform).

Results

Effect of herbivore feeding site on pollinator behavior

We observed the behavior of 908 responsive pollinators, with 182 h of observation time, and about 9,000 flower visits. The behavior of syrphid flies and butterflies was influenced by herbivore infestation, and the effects depended on herbivore and pollinator

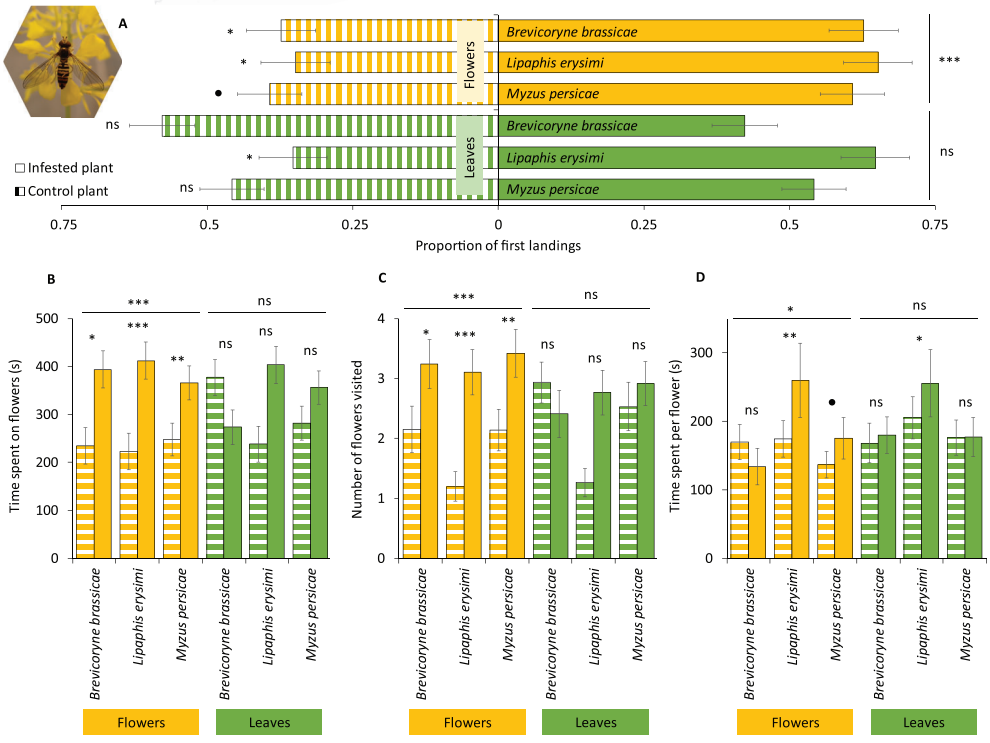


Fig. 1 Preference of the syrphid fly *Episyrphus balteatus* for uninfested *Brassica nigra* plants or plants infested with herbivores on leaves or flowers. A) Proportion of *E. balteatus* syrphid flies (mean ± SE) that first landed on flowers or leaves of *B. nigra* plants infested with herbivores on leaves or flowers, or uninfested plants. B) Visitation time (mean ± SE), C) number of flowers visited (mean ± SE), D) and time spent per flower (mean ± SE) by individual pollinators on infested or uninfested *B. nigra* plants. Number of replicates per herbivore treatment varied between 66 and 83 syrphid flies, and 7 and 9 plant pairs. Asterisks above bars indicate significant differences with *** = $P < 0.001$, ** = $0.001 \geq P < 0.01$, * = $0.01 \geq P \leq 0.05$, and ● = $0.05 > P < 0.1$, based on Tukey's *post hoc* tests. Picture shows an *E. balteatus* syrphid fly visiting flowers of *B. nigra*. Photograph credits: Quint Rusman

species, as well as the feeding site of the herbivore. Overall, syrphid flies landed more frequently on flower-infested plants when compared with uninfested plants (Fig. 1, GLMM: z = 3.67, $P < 0.001$). This was true for plants infested with *L. erysimi* (GLMM: z = 2.42, $P = 0.015$), *B. brassicae* (GLMM: z = 2.05, $P = 0.040$), and was marginally significant for *M. persicae* (GLMM: z = 1.90, $P = 0.058$). In contrast, folivory did not influence the preference of *E. balteatus* and syrphid flies landed as frequently on infested plants as they did on uninfested plants (Fig. 1, GLMM: z = 1.00, $P = 0.322$). This was indeed the case for plants infested with *B. brassicae* (GLMM: -z = 1.35, $P = 0.176$), and *M. persicae* (GLMM: z = 0.77, $P = 0.443$). However, syrphid flies landed more frequently on plants infested with *L. erysimi* compared with uninfested plants (GLMM: z = 2.39,

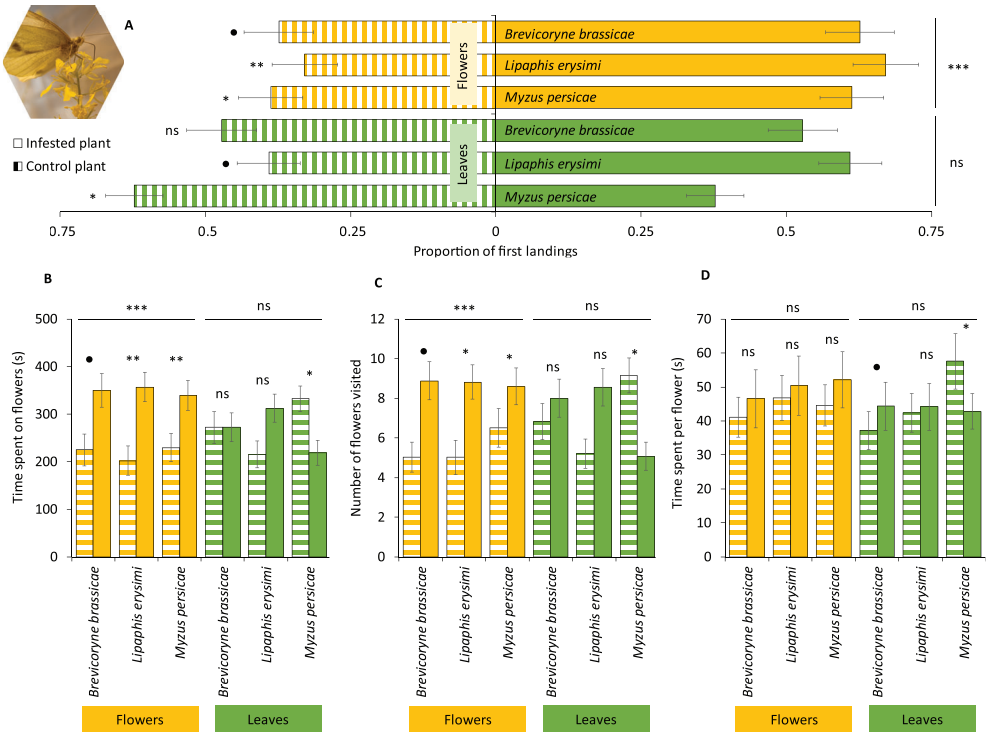


Fig. 2 Preference of the butterfly *Pieris brassicae* for uninfested *Brassica nigra* plants or plants infested with herbivores on leaves or flowers. A) Proportion of *P. brassicae* butterflies (mean ± SE) that first landed on flowers or leaves of *B. nigra* plants infested with herbivores on leaves or flowers, or uninfested plants. B) Visitation duration (mean ± SE), C) number of flowers visited (mean ± SE), D) and time spent per flower (mean ± SE) by individual pollinators on infested or uninfested *B. nigra* plants. Number of replicates per herbivore treatment varied between 67 and 98 butterflies, and 7 and 10 plant pairs. Asterisks above bars indicate significant differences with *** = $P < 0.001$, ** = $0.001 \geq P < 0.01$, * = $0.01 \geq P \leq 0.05$, and ● = $0.05 > P < 0.1$, based on Tukey's *post hoc* tests. Picture shows a *P. brassicae* butterfly visiting flowers of *B. nigra*. Photograph credits: Quint Rusman

$P = 0.017$). For most treatments, herbivore infestation and feeding site had the same effect on the duration of visitation and the number of flowers visited as for the landing preference for syrphid flies (Fig. 1). Syrphid flies spent more time per flower on flower-infested plants when compared with uninfested plants (Fig. 1, GLMM: df = 1, $\chi^2 = 4.72$, $P = 0.030$). This was recorded for plants infested with *L. erysimi* (GLMM: df = 1, $\chi^2 = 10.77$, $P = 0.001$), marginally significant for *M. persicae* (GLMM: df = 1, $\chi^2 = 3.57$, $P = 0.059$), but not for plants infested with *B. brassicae* (GLMM: df = 1, $\chi^2 = 2.49$, $P = 0.115$). Syrphid flies spent similar amounts of time per flower when offered a choice between flowers from leaf-infested and uninfested plants (Fig. 1, GLMM: df = 1, $\chi^2 = 2.27$, $P = 0.132$). However, infestation with *L. erysimi* influenced their choice. Syrphid flies spent

more time per flower of plants infested with *L. erysimi* on leaves than on uninfested plants (GLMM: df = 1, χ^2 = 4.61, *P* = 0.032). In general, visitation of syrphid flies on infested plants was not affected by aphid abundance (Table D1), except for visitation duration on flower-infested plants with *L. erysimi* (τ = -0.27, z = -2.43, *P* = 0.015).

Overall, butterflies landed more frequently on flower-infested plants when compared with uninfested plants (Fig. 2, GLMM: z = 3.95, *P* < 0.001). This was true for plants infested with *L. erysimi* (GLMM: z = 2.81, *P* = 0.005) and *M. persicae* (GLMM: z = 2.00, *P* = 0.046), and only marginally significant when regarding *B. brassicae* (GLMM: z = 1.76, *P* = 0.079). Overall, folivory did not influence *P. brassicae* choice, and butterflies landed as frequently on leaf-infested plants as they did on uninfested plants (Fig. 2, GLMM: z = -0.02, *P* = 0.981). This was indeed recorded when considering only plants infested with *B. brassicae* (GLMM: z = 0.367, *P* = 0.714), or *L. erysimi* (GLMM: z = 1.66, *P* = 0.096). However, butterflies landed less frequently on plants infested with *M. persicae* compared with uninfested plants (GLMM: z = -2.40, *P* = 0.016). For most treatments, herbivore infestation and feeding site had the same effect on the duration of visitation and the number of flowers visited as for the landing preference for butterflies (see Fig. 2). Butterflies spent similar amounts of time per flower irrespective of the herbivore species or feeding site in plant treatments, except for plants infested with *M. persicae* on the leaves. In the latter case, butterflies spent more time per flower when compared with uninfested plants (GLMM: df = 1, χ^2 = 6.14, *P* = 0.013). In general, visitation of butterflies on infested plants was not affected by aphid abundance (Table D1), except for time spent per flower on leaf-infested plants with *L. erysimi* (cor = -0.29, t = -2.22, *P* = 0.031).

Herbivore preference for leaves or flowers

All three aphid species were recorded more frequently on inflorescences than on vegetative tissues (Fig. 3, Tukey's *post hoc* tests; *B. brassicae*: *P* < 0.001, *L. erysimi*: *P* < 0.001, *M. persicae*: *P* < 0.001). Behavioral choices, for various plant organs within either inflorescence - buds, flowers, floral stems, bracts - or vegetative parts - stems, young leaves, old leaves - differed depending on the aphid species (Fig. 3, GLM: χ^2 = 83.42, df = 6, *P* < 0.001). Overall, most winged aphids were recorded among buds (Fig. 3, GLM: χ^2 = 141.77, df = 12, *P* < 0.001). When comparing the different species, relatively more winged *B. brassicae* aphids were recorded on buds and bracts compared with numbers of *L. erysimi* (Tukey's *post hoc* tests, buds: *P* = 0.012; bracts: *P* = 0.007) and *M. persicae* (Tukey's *post hoc* tests, buds: *P* = 0.001; bracts: *P* = 0.029). Fewer winged aphids of *B. brassicae* were found on flowers and floral stems compared with numbers of *L. erysimi* (Tukey's *post hoc* tests, flowers: *P* < 0.001; floral stems: *P* = 0.001) and *M. persicae* (Tukey's *post hoc* tests, flowers: *P* < 0.001; floral stems: *P* < 0.001).

Herbivore performance on leaves or flowers

Aphid performance was affected by feeding site (GLM: $\chi^2 = 33.89$, df = 1, $P < 0.001$) and aphid species, resulting in a significant interaction between these two factors (GLM: $\chi^2 = 39.83$, df = 2, $P < 0.001$). Overall, aphids performed better on flowers than on leaves (Fig. 4, Tukey's *post hoc* test, $P = 0.041$). This was the case for *B. brassicae* and *L. erysimi* (Tukey's *post hoc* tests, $P = 0.041$ and $P < 0.001$ respectively), whereas *M. persicae* performed equally well on both plant tissues (Tukey's *post hoc* test, $P = 0.543$). The magnitude of effect of feeding site was strongest for *L. erysimi*, which had 2.4 times more individuals on flowers than on leaves.

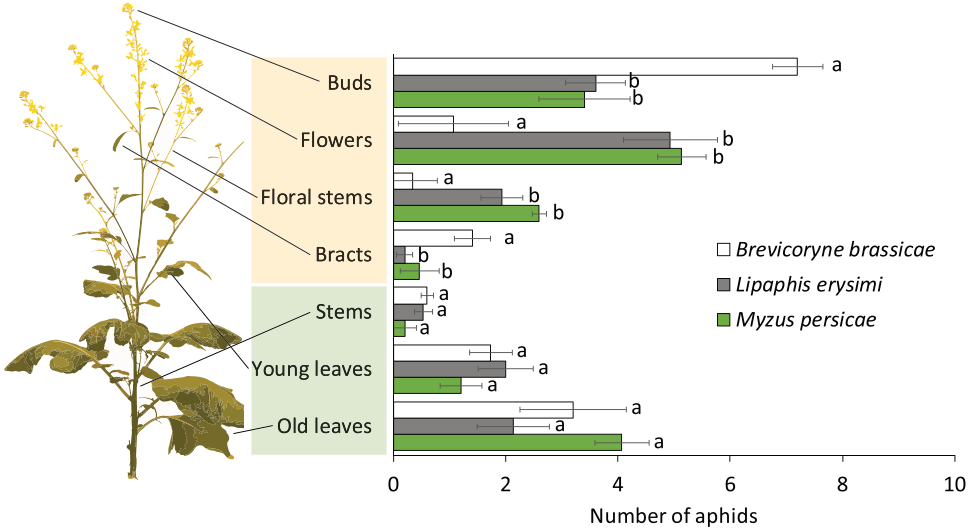


Fig. 3 Numbers of winged aphids of three different species (Mean ± SE) on various plant organs of *Brassica nigra* plants. The position of 20 winged aphids was determined 24 hours after release. Number of plant replicates was 15 for each aphid species. Letters indicate significant differences at $\alpha = 0.05$ based on Tukey's *post hoc* tests when comparing differences between species within a plant organ. Photograph credits: Dani Lucas-Barbosa.

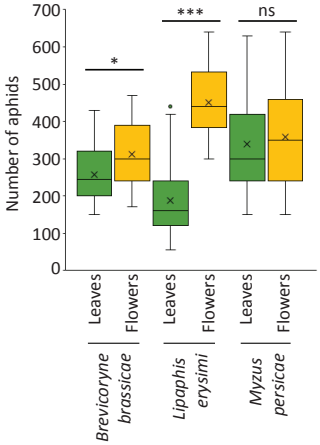
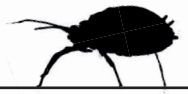


Fig. 4 Numbers of aphids of three different species on leaves and flowers of *Brassica nigra* plants. Adult aphids and nymphs were counted 7 days after infestation. Boxplots show median (line), mean (x), 1st and 3rd quartiles, minimum and maximum. Outliers are represented by circles (1.5 times the interquartile range below the 1st or above the 3rd quartile). Asterisks above lines indicate significant differences between feeding sites for each aphid species based on Tukey's *post hoc* tests, where * = $0.01 \leq P \leq 0.05$ and *** = $P \leq 0.001$. Number of plant replicates varied between 23 and 25.



Discussion

Our data show that indirect plant-mediated interactions between antagonists and mutualists are dependent on the feeding-site chosen by plant antagonists. Florivory positively affected pollinator visitation, independent of the pollinator or aphid species tested. In contrast, folivory had limited effects on pollinator visitation. Flowering plants may experience florivory more often than folivory because all three herbivore species preferred to settle on flowers over leaves. The choice of feeding site matched the performance for most herbivores; the two specialist aphids performed better on flowers than on leaves, whereas the generalist aphid performed equally well on both plant parts. Taken together, the choice of the feeding site by the adult herbivores maximizes the species performance and had profound impact on the outcome of indirect interactions between herbivores and pollinators. Hence, the evolution of antagonist feeding behavior might affect mutualist community assembly *via* trait-mediated interactions.

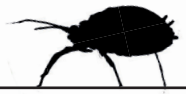
The importance of herbivore feeding site as determinant of plant-mediated interactions can be explained by differences in plant responses to herbivores that chose different feeding sites. Indeed, plants respond differentially to the same herbivore species when feeding on different feeding sites, such as leaves and flowers (Farré-Armengol et al. 2015), leaves and roots (Hladun and Adler 2009, Barber et al. 2011), or leaves of different ages (Bingham and Agrawal 2010, Quintero and Bowers 2011). This is most likely caused by tissue-specific plant responses (Utsumi and Shefferson 2015, Johnson et al. 2016, Chrétien et al. 2018). Alternatively, plants may respond differentially because of differences in herbivore damage or performance, which may subsequently affect plant-mediated interactions (Kroes et al. 2017, Pineda et al. 2017). Although both specialist aphids performed better on flowers than on leaves, the generalist aphid performed equally well on flowers and leaves. Still, florivory by *M. persicae* positively affected pollinator visitation, whereas folivory had no or even a negative effect. This suggests tissue-specific plant responses rather than density-dependent plant responses. Hence, the importance of herbivore feeding site as determinant for trait-mediated interactions is likely mediated by plant responses that are tissue-specific (Utsumi and Shefferson 2015).

In addition to indirect trait-mediated interactions, florivore-induced changes in pollinator attraction can be mediated indirectly by interaction modification. During indirect interaction modification, a species modifies how two other species interact without affecting either of the two species directly (Wootton 1994). For example, aquatic invertebrates interact indirectly with limpets by reducing bird predation *via* crypsis because limpets were harder to find for bird predators among mussels and barnacles (Wootton 1992). Florivore presence can change how flower traits are perceived by flower visitors without affecting the trait itself. Here, the presence of the aphids could have enhanced the visual appearance of the flowers, by enhancing contrast with the

background, leading to an increase in pollinator attraction. Alternatively, the aphids themselves could have attracted the pollinators, which would then spill over to the plant (Almohamad et al. 2009). This might be the case for syrphid flies, which larvae are common natural enemies of all three aphid species, but less so for butterflies, which larvae are herbivores themselves. More importantly, most inflorescences of herbivore-infested plants remained free of herbivores, and we never observed that pollinators were particularly attracted or repelled by herbivore-infested inflorescences or the presence of the herbivores themselves in the greenhouse or field (pers. obs. Quint Rusman and Peter Karssemeijer). Thus, the herbivore-pollinator interactions observed in this study were most likely trait-mediated indirect interactions.

Antagonist feeding site can be important for the plant fitness consequences of trait-mediated indirect interactions. An increase in visitation by pollinators due to florivory might result in a positive net effect on plant reproduction. This is in fact supported by field evidence, where plant responses to belowground herbivores lead to increased plant reproduction, whereas plant responses to aboveground herbivores did not (Rusman et al. 2018 - chapter 3). Herbivore species that are more likely to affect plant fitness indirectly through plant-mediated interactions with antagonistic or mutualistic members of the plant-associated community can be considered keystone herbivore species in influencing trait evolution of a given plant species (Poelman and Kessler 2016). Because of the important consequences of feeding preference for plant-mediated antagonist-mutualist and antagonist-antagonist interactions, feeding site might be an important characteristic of keystone herbivores. An exciting hypothesis that remains to be investigated in a single manipulative experiment is whether early colonizing keystone herbivores feeding on leaves or roots mainly affect plant fitness *via* effects on herbivore and/or natural enemy communities (Pashalidou et al. 2015), whereas later arriving keystone herbivores feeding on flowers affect plant fitness *via* altering the composition of flower visitor communities (Rusman et al. 2018 - chapter 3).

The increased visitation by pollinators due to florivory-induced plant responses can be a plant strategy to optimize reproduction, or an ecological consequence of the defenses induced by florivores. Accelerating seed production indirectly *via* enhanced pollinator attraction may be a way for plants to safeguard reproduction (Lucas-Barbosa et al. 2013, Rusman et al. 2018 - chapter 3). The increased attraction of pollinators might also be a consequence of the tight intrinsic linkage between defense and reproduction (Johnson et al. 2015, Lucas-Barbosa 2016). For example, floral volatiles mediate interactions with pollinators and natural enemies of herbivores (Schiestl et al. 2014). Pigments color the flower, and can be toxic compounds for herbivores while being exploited by pollinators during foraging (Johnson et al. 2015). Plants may respond to herbivore attack with changes in floral volatiles to attract natural enemies, or by increasing concentrations of pigments to enhance resistance to herbivores (Schiestl et al. 2014, Boyer et al.



2016). Changes in flower traits can subsequently mediate indirect interactions between herbivores and pollinators, and may make plants more attractive to certain pollinators, but less to others (Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3). The complete network of indirect plant-mediated interactions between herbivores and flower visitors (both mutualists and antagonists) will determine the consequences for plant reproduction (Poelman and Kessler 2016, Rusman et al. 2018 - chapter 3). When consequences for plant reproduction are effectively neutral, herbivore-induced changes in flower traits are maintained as part of the plant's inducible defensive responses (Gould and Lewontin 1979), whereas positive or negative consequences will impose selection on plant defense and/or reproduction (Johnson et al. 2015, Lucas-Barbosa 2016, Poelman and Kessler 2016).

The impact of feeding site choice on herbivore performance, and on the outcome of plant-mediated interactions between antagonists and mutualists can create an eco-evolutionary feedback loop (Ohgushi 2016). Antagonist feeding site choice differently affected mutualist visitation, which might cascade and influence mutualist network assembly, thereby affecting plant performance and reproduction, and subsequently selection on plant traits, which will in turn feedback on the antagonists by affecting antagonist performance, and creating potential for selection on feeding site choice (Fig. 5). This results in an eco-evolutionary feedback loop between the mutualist network and the evolution of antagonist feeding preference, *via* inducible plant responses. Indeed, plant-mediated interactions between herbivores and pollinators may cascade to affect pollinator community assembly and plant reproduction (Steets et al. 2006, Ghyselen et al. 2016, Hoffmeister et al. 2016, Chautá et al. 2017, Rusman et al. 2018 - chapter 3). With herbivore feeding preference as main determinant of trait-mediated antagonist-mutualist interactions, herbivore feeding site choice will affect mutualist network assembly. The effect of feeding preference on community dynamics *via* trait-mediated interactions has so far only been shown for antagonist community members (Johnson et al. 2009, Farkas et al. 2013, Utsumi and Shefferson 2015, Matthews et al. 2016). Herbivore-induced changes in pollinator community composition and plant reproduction likely impose selection on flower traits and plant defense (Johnson et al. 2015, Lucas-Barbosa 2016, Poelman and Kessler 2016), which could lead to rapid evolution of plant traits (Schiestl and Johnson 2013, Gervasi and Schiestl 2017). Changes in plant traits may in turn affect the feeding preference of the herbivores (McCall and Irwin 2006, Strauss and Whittall 2006, McCall et al. 2013), completing the eco-evolutionary feedback loop. A similar eco-evolutionary feedback loop through inducible plant responses seems to affect the evolution of feeding preference of leaf beetles on willow and its associated arthropod community (herbivores and predators) (Utsumi and Shefferson 2015). Thus, trait-mediated interactions involving both antagonistic and mutualistic organisms can potentially play an important role in eco-evolutionary dynamics.

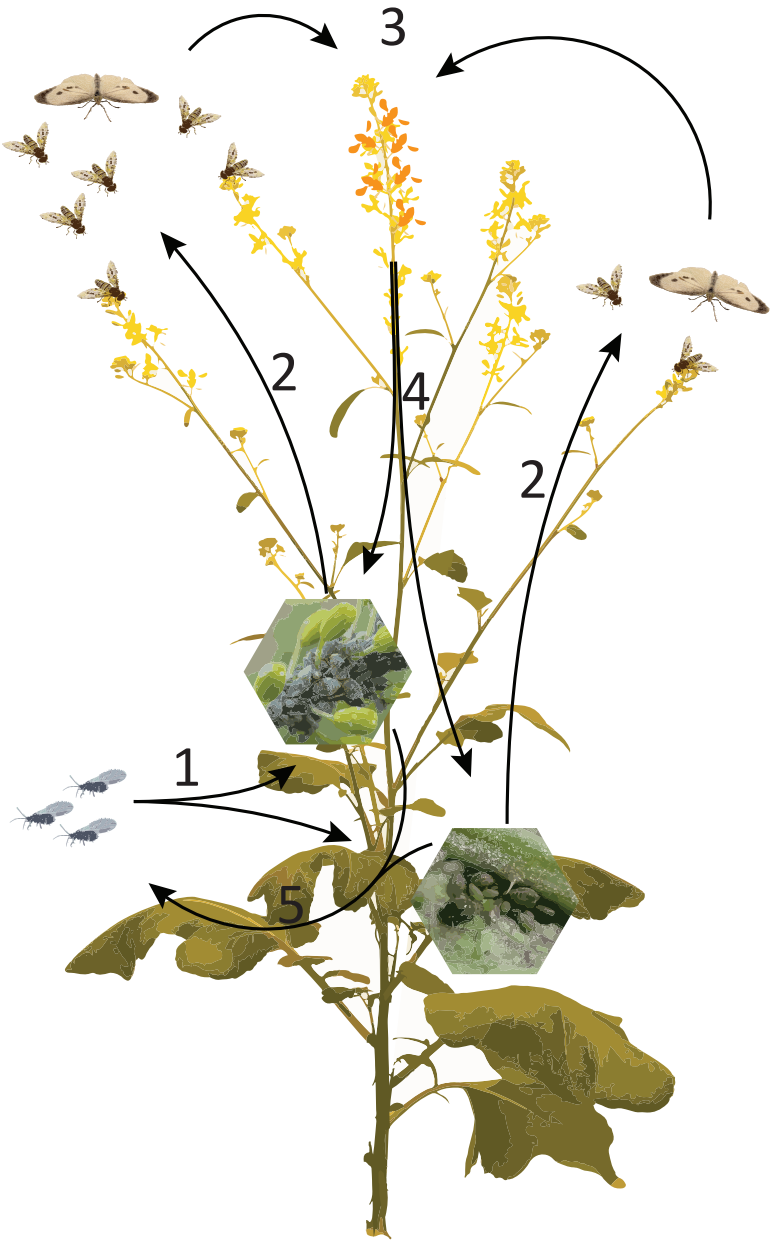
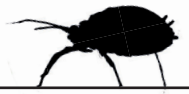


Fig. 5 Illustration of the eco-evolutionary feedback loop *via* trait-mediated interactions between plant antagonists and mutualists. Antagonists choose a feeding site that maximizes its performance (1); Feeding site choice differentially affects herbivore-induced plant responses and interactions with mutualists, which will cascade to alter mutualist network assembly (2); Herbivore-induced changes in mutualist network assembly imposes selection on plant traits such as defence and/or reproductive traits (3);. Changes in plant traits affect antagonist performance (4), which subsequently imposes selection on antagonist feeding behaviour (5). Photograph credits: Dani Lucas-Barbosa and Quint Rusman.



Our study shows the potential through which trait variation may affect species interactions *via* trait-mediated interactions. This can subsequently affect ecological community organization, which in turn may affect trait evolution (Ohgushi 2016, Poelman and Kessler 2016), especially if traits are evolutionary labile and can evolve rapidly in response to variation in the biotic environment, such as feeding preferences of herbivores (Thompson 1998). Such eco-evolutionary feedback loops might be widespread in nature (Utsumi 2011, Utsumi and Shefferson 2015), and involve mutualists in addition to antagonists. Our study contributes to the growing appreciation of the role of trait-mediated interactions in the ecology and evolution of communities, and our work identifies that antagonist trait variation can be important in determining the linkage between antagonistic and mutualistic plant-associated communities. Understanding of such eco-evolutionary dynamics is critical for our understanding of the ecological and evolutionary outcome of species interactions in plant-associated communities.

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Chapter 7

General discussion:

Eco-evolutionary dynamics of plant-mediated herbivore - flower-visitor interactions

Introduction

Explaining patterns of biodiversity around the globe is one of the supreme challenges for ecologists. By studying interaction networks, such as food webs, ecologists try to understand how organisms survive and interact with each other. In multispecies communities we typically observe indirect interactions in addition to direct interactions (Wootton 1994). Indirect interactions occur when the interaction of two organisms is mediated through another organism. For example, kangaroo rats (*Dipodomys* spp.) prefer to consume seeds of plant species with large seeds and thereby indirectly increase the abundance of plant species with small seeds. This indirectly increases the abundance of ants because they forage more effectively on small seeds, and seed-eating birds because kangaroo rats reduce vegetation cover which allows birds to more easily detect seeds (Davidson et al. 1984, Thompson et al. 1991). Such indirect interactions can have considerable impact on community composition and dynamics (Werner and Peacor 2003, Schmitz et al. 2010), but also on evolutionary changes and trait distribution in communities (Strauss et al. 2005, Turcotte et al. 2012, Guimarães Jr et al. 2017). As a result, there seems to be a profound interplay between ecological and evolutionary dynamics of communities.

Plant-associated communities are packed with indirect interactions, including interactions between antagonists and mutualists. Plants need to deal with antagonists such as herbivores, while engaging in interactions with mutualists such as pollinators to maximize reproductive output. Most plants respond to attack by antagonists with phenotypic changes to repel or kill the attackers (Karban and Baldwin 1997). Such plant phenotypic changes in response to antagonist attack are often systemically expressed throughout the plant (Hilleary and Gilroy 2018). Plant phenotypic changes do not only affect the attacker, but may alter interactions with other community members as well. For instance, when plants are flowering, antagonist attack to leaves or roots can induce phenotypic changes in flowers (Barber and Gorden 2015, Lucas-Barbosa 2016, Jacobsen and Raguso 2018). Such herbivore-induced changes in flower traits occur because reproductive and resistance traits are physiologically linked (Rusman et al. chapter 2). As a result, herbivore attack can alter interactions of plants with pollinators and flower feeders. Plant responses to herbivores may have negative, positive, or neutral effects on the visitation and performance of mutualistic and antagonistic flower visitors (Kessler and Halitschke 2009, McArt et al. 2013, Lucas-Barbosa et al. 2016, McCall et al. 2018). It is currently unclear how specific interactions between herbivores and flower visitors are, and whether specificity in plant responses to herbivory extend to flower-trait expression. Moreover, indirect interactions seem important for the assembly of antagonist communities on plants (Poelman et al. 2008, Poelman et al. 2010, Stam et al. 2018), and recent syntheses of coevolutionary processes argue for a profound role

of indirect interactions in plant-antagonist networks (Poelman and Kessler 2016) as well as plant-mutualist networks (Guimarães Jr et al. 2017). Because both antagonists and mutualists influence the reproductive output of a plant (Herrera et al. 2002, Grass et al. 2018), altered interactions of plants with antagonists and mutualists as a result of herbivory can have consequences for plant reproduction (Poelman and Kessler 2016). Knowledge on the importance of indirect interactions for the assembly of flower-visitor networks and associated consequences for plant fitness is limited to date.

The aim of this thesis project was to address specificity of plant-mediated (indirect) interactions between antagonists and mutualists, and assess potential fitness consequences for the plant. My research explored whether and how flowering plants respond to attack by different herbivores, whether and how this affects interactions with mutualistic pollinators and antagonistic flower feeders, and plant reproductive output. Here, I discuss the implications of my results and place the findings of my work in an ecological and evolutionary framework.

Unravelling patterns in plant-mediated herbivore - flower-visitor interactions

Plant-mediated herbivore-pollinator interactions are part of the set of interactions that shape most plant-associated communities. For annual plant species with diverse herbivore communities and a generalized pollination system, herbivore attack can affect plant interactions with pollinators (Steets et al. 2006, Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3). Biannual and perennial herbs can also mediate herbivore-pollinator interactions (Cardel and Koptur 2010, Kessler et al. 2011), although this does not always seem to be the case (Burkle and Runyon 2016, Hernandez-Cumplido et al. 2016). For longer-lived plants like shrubs and trees we have limited examples, which do suggest that herbivory can alter pollinator visitation (Parra-Tabla and Herrera 2010, Tsuji et al. 2016, Chautá et al. 2017, Tsuji and Ohgushi 2018).

A large variety of herbivores have been shown to affect flower visitors *via* herbivore-induced plant responses. Examples from a variety of study systems include generalist and specialist herbivores, members of different feeding guilds, such as chewing and sap-feeding insects, that may each feed on different plant parts such as roots, leaves, and flowers (Hambäck 2001, Kessler and Halitschke 2009, Schiestl et al. 2014, Ikemoto et al. 2017). However, comparative approaches which assess plant responses to different herbivores and effects on flower traits and flower visitors within the same study system have rarely been employed. Such approaches could tell us how common herbivore - flower-visitor interactions are within communities, and how these vary among different

herbivore species. The data presented in this thesis show that of the about 30 herbivore species that colonize *Brassica nigra* in the field, plant infestation with each of the ten herbivore species studied here affected visitation by flower visitors (Rusman et al. 2018 - chapter 3, Rusman et al. 2019 - chapter 5, Rusman et al. chapter 4, 6). Herbivores varying in feeding guilds and feeding sites (aboveground chewing and sap-feeding herbivores and root-feeding herbivores) have different effects on visitation by different pollinator functional groups (bumblebees, syrphid flies, solitary bees) (Fig. 1) (Rusman et al. 2018 - chapter 3). Individual herbivore-pollinator interactions seem specific for the herbivore species that damages the plant (Rusman et al. 2019 - chapter 5), except for sap-feeding herbivores, where feeding site (leaves or flowers) is an important determinant of effects on pollinators (Fig. 2) (Rusman et al. chapter 6). Moreover, the outcomes of specific herbivore - flower-visitor interactions depend on the plant ontogenetic stage in which the herbivore attacks the plant (Fig. 1) (Rusman et al. chapter 4). Overall, this thesis shows the potential of each individual herbivore species within an herbivore community to induce plant-mediated interactions with antagonistic and mutualistic flower visitors.

Flower visitors readily respond to herbivore-induced changes in plant phenotype. When visitation by communities of generalist pollinators is considered, the total number of pollinator visits can be negatively affected by herbivore-induced plant responses (Steets et al. 2006, Rusman et al. chapter 4), or remain equal if compared with uninfested plants (Lucas-Barbosa et al. 2013, Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3). Although total number of pollinators can remain equal, the composition of the pollinator community may change, indicating that visitation by some pollinator species increases, whereas visitation by other species decreases (Steets et al. 2006, Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3). Plant infestation with herbivores can alter the visitation rates by various generalist pollinators, such as honeybees, bumblebees, syrphid flies, and butterflies (Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3, Rusman et al. 2019 - chapter 5, Rusman et al. chapters 4, 6). Specialized pollinator systems have received relatively less attention in this perspective. It remains to be investigated if pollinators that are specialized on flowers of a single or few plant species respond as often to herbivore-induced changes in flower traits as generalist pollinators do. Antagonistic flower visitors like florivores, seed predators, nectar thieves and robbers are mostly negatively affected by plant phenotypic changes in response to herbivory on leaves or flowers (McArt et al. 2013, Boyer et al. 2016, Soper Gorden and Adler 2016, McCall et al. 2018, Rusman et al. chapter 4). Antagonistic flower visitors may either avoid herbivore-infested plants, or have a reduced performance on herbivore-infested compared with uninfested plants (McCall 2006, McArt et al. 2013, Ikemoto et al. 2017, McCall et al. 2018). In a few cases, plants that were attacked by root or leaf herbivores or received artificial flower damage were more attractive to florivores (Soper Gorden and Adler 2016, Rusman et

al. *unpublished*). Responses of antagonistic flower visitors to herbivore-induced changes in plant phenotype can cascade to affect florivore community composition (Stam et al. 2018). Taken together, herbivore - flower-visitor interactions seem widespread among different ecosystems, including a variety of different plants, herbivores, and mutualistic and antagonistic flower visitors.

Trait variation in herbivore - flower-visitor interactions

An important driver of ecological dynamics resulting from species interactions is trait variation. Species traits determine an organism's niche width, and thereby interactions with other species. Trait variation can alter the number and strength of an organism's interspecific species interactions, which subsequently changes the structure and dynamics of species-interaction networks (Bolnick et al. 2011). Indeed, trait variation in indirect interactions can have community-wide consequences (Johnson et al. 2009, Farkas et al. 2013, Matthews et al. 2016, Ohgushi 2016). For example, variation in feeding-site preference of leaf beetles for young or old willow leaves induces different plant responses, with different effects on the composition of the arthropod community, including herbivores and predators (Utsumi and Shefferson 2015). Still, a critical knowledge gap in understanding ecological dynamics in interactions between antagonists and mutualists is the role of trait variation in such interactions.

In this thesis, I show that for herbivore - flower-visitor interactions trait variation among the different interactors - herbivores, plants, and flower visitors - is crucial and affects the behaviour of flower visitors and the dynamics of a flower visitor community (Fig. 1 + 2). Interspecific variation in herbivore feeding guild and feeding site influences changes in visitation by pollinator functional groups (Rusman et al. 2018 - chapter 3), whereas individual herbivore - flower-visitor interactions are largely herbivore-species-specific (Rusman et al. 2019 - chapter 5). Intraspecific variation in herbivore feeding position can lead to similar effects of different herbivores on pollinator visitation: florivory by different aphid species can increase the attraction of pollinators, whereas folivory can have variable effects from increased attraction to repellence of pollinators (Rusman et al. chapter 6). Plant ontogeny increases specificity of effects on pollinator and florivore visitation: the direction and strength of individual herbivore - flower-visitor interactions varies depending on the plant ontogenetic stage during which herbivore attack occurs (Rusman et al. chapter 4). Thus, trait variation plays a crucial role in plant-mediated interactions between antagonists and mutualists.

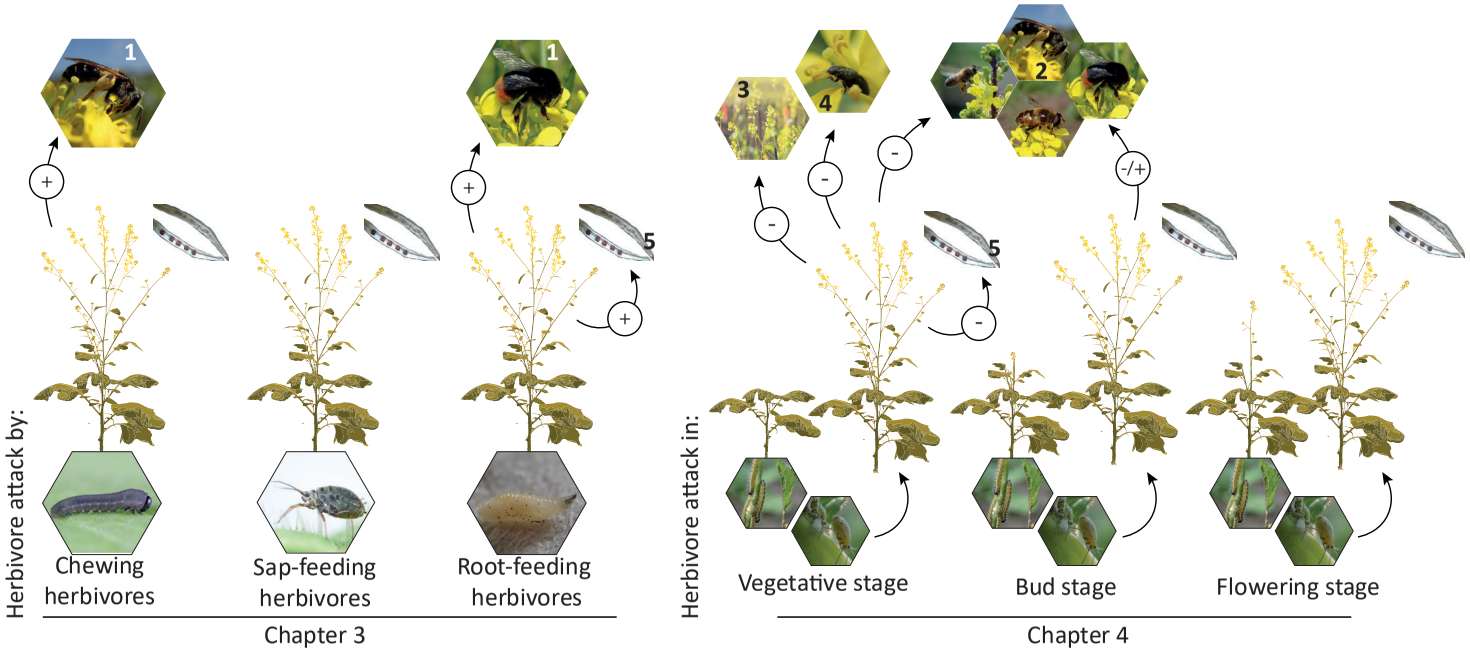

Fig. 1 Schematic representation that summarizes the main results of chapters 3 and 4 where flowering *Brassica nigra* (Chapter 3; left panel) or 3 different ontogenetic stages of *B. nigra* (Chapter 4; right panel) were exposed to different herbivores and the effects on mutualistic and antagonistic flower visitors and seed production investigated. Arrows indicate an effect on the visitation of specific pollinator groups (1) or all pollinators (2), number of flowers (3), abundance of pollen beetles (*Meligethes* spp.) (4), and plant seed production (5). The sign in the circle represents the direction of effect when compared with uninfested plants, where positive effects (+), and negative effects (-) were quantified. No arrow indicates no significant effects. Photograph credits: Jitte Groothuis, Dani Lucas-Barbosa, and Quint Rusman.

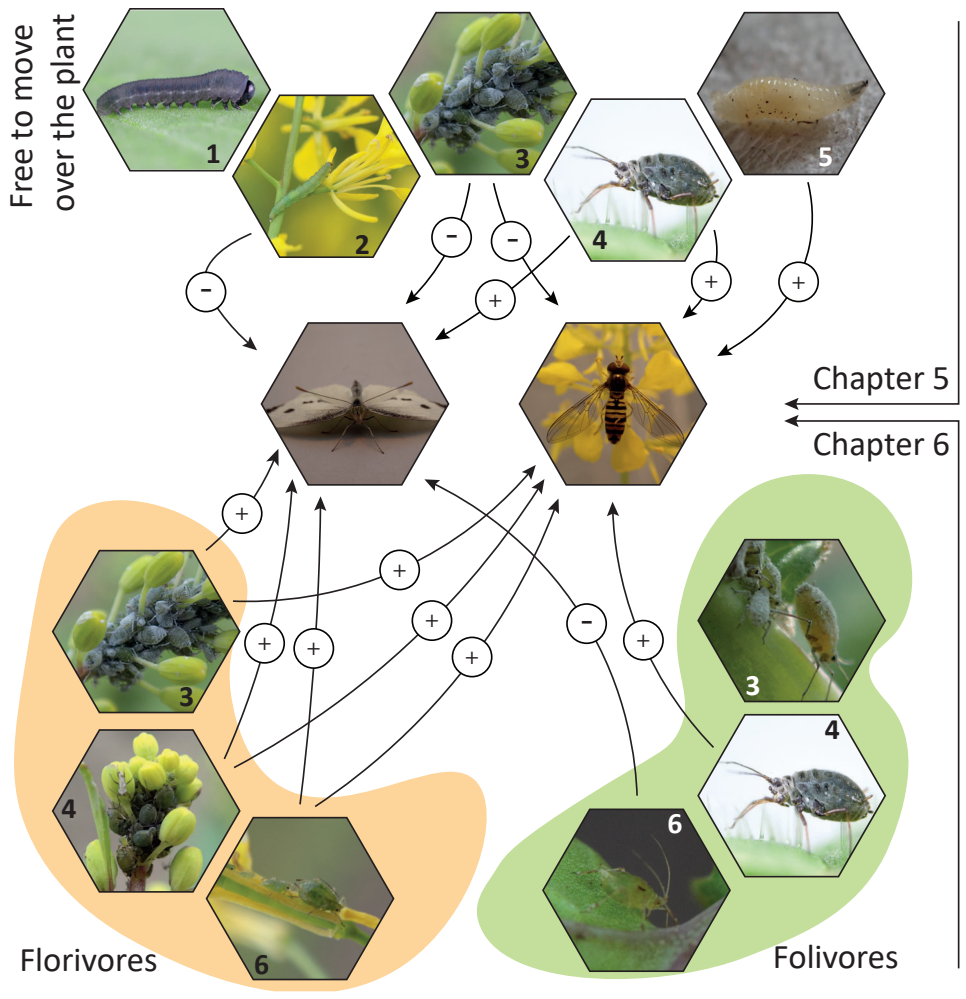

Fig. 2 Schematic representation that illustrates the main results of chapter 5 and 6 where effects of herbivory on flower visitation by butterflies (*Pieris brassicae*) and syrphid flies (*Episyrphus balteatus*) were investigated. The effects of *B. nigra* exposure to various herbivore species (Chapter 5; top panel) and to aphid species when feeding on leaves or flowers (Chapter 6; bottom panel) on pollinator visitation are shown by arrows. The sign in the circle represents the direction of effect as compared to uninfested plants, where + = positive effects, and - = negative effects. No arrow indicates no significant effects. Herbivores are 1) *Athalia rosae*, 2) *Plutella xylostella*, 3) *Brevicoryne brassicae*, 4) *Lipaphis erysimi*, 5) *Delia radicum* and 6) *Myzus persicae*. Photograph credits: Jitte Groothuis, Dani Lucas-Barbosa, and Quint Rusman.

Trait variation in antagonistic inducers

Herbivores adopted a wide variety of different feeding behaviours, including different feeding guilds, feeding positions, and host-plant ranges to either circumvent or take advantage of plant resistance traits (Bernays 1998). In return, plants evolved various defensive strategies, including inducible defences, to successfully defend against different herbivores. Inducible defences allow plant responses to be fine-tuned to the specific attacker, save metabolic costs of resistance in the absence of herbivores, reduce the opportunities for herbivores to adapt to a fixed plant phenotype, and facilitate information exchange between the plant and its environment (Karban and Baldwin 1997, Karban 2011, Kessler 2015). Feeding behaviour of the herbivore is important in determining a general component in plant responses to herbivory, whereas a more specific component is based mostly on herbivore identity (Erb et al. 2012). Herbivore feeding guild is important for the elicitation of plant responses in leaves (Ali and Agrawal 2012, Thaler et al. 2012), and these plant responses are regulated by phytohormonal signal-transduction pathways (Pieterse et al. 2012). In general, chewing herbivores induce the jasmonic acid (JA) pathway, whereas sap-feeding herbivores usually induce the salicylic acid (SA) pathway and/or suppress the JA pathway. Belowground chewing herbivores also induce the JA pathway but they induce different plant responses compared to aboveground chewing herbivores (Johnson et al. 2016). This is, among others, due to differences in the belowground phytohormonal network (Lu et al. 2015). We might therefore expect that herbivores from similar feeding guilds/sites induce more similar plant responses compared to herbivores from different feeding guilds/sites. I have shown that for flower traits this is not true: herbivore-induced changes in most flower traits are herbivore species-specific (Rusman et al. 2019 - chapter 5). Differences in specificity of herbivore-induced changes in leaves and flowers may be the result of plant-tissue-specific and plant-ontogeny-specific expression of genes and regulatory components resulting in differential expression of plant responses (Rusman et al. chapter 2). Also, phytohormonal networks are more complex than the common contrast usually illustrated between JA- and SA pathways. Many other phytohormones such as ethylene and abscisic acid modulate the JA- and SA-mediated responses and some can function as JA- and SA-independent pathways (Erb et al. 2012). Therefore, differences in elicitation of the JA- and SA pathways may not accurately predict differences in induced resistance or flower traits. Still, herbivore traits are important in determining plant phenotypic changes, and we expect feeding behaviour by antagonists to provide important trait variation in interactions between antagonists and mutualists.

Trait variation in antagonist-antagonist interactions

Variation in herbivore feeding guild and feeding site may provide important trait variation

that influences the outcome of plant-mediated antagonist-antagonist interactions. Indirect interactions between herbivores from different feeding guilds feeding on leaves of the same plant have been relatively well explored. For example, feeding by caterpillars may negatively affect the preference and performance of subsequently colonizing chewing herbivores, whereas aphids may have positive or neutral effects (Rodriguez-Saona et al. 2005, Rodriguez-Saona et al. 2010, Soler et al. 2012, Eisenring et al. 2018). *Vice-versa*, caterpillars may positively affect the performance of aphids (Soler et al. 2012), whereas other aphids may have predominantly negative effects on aphid performance (Züst and Agrawal 2016). However, the feeding guild of antagonists cannot always predict the outcome of such interactions, and overall patterns show no clear difference in the outcome of intra- and interguild competition (Kaplan and Denno 2007). Indeed, the outcome of antagonist-antagonist interactions with inducers from the same feeding guild can be variable, as shown for chewing herbivores (Agrawal 2000b) and sap-feeding herbivores (Zhang et al. 2015). This is caused by additional variation in herbivore traits other than feeding guild that modulate plant responses to herbivory and subsequent plant-mediated interactions beyond herbivore feeding guild, such as herbivore feeding site (de Rijk et al. 2016, Rusman et al. chapter 6).

When plants start flowering, new organs are produced on which herbivores can feed. Many herbivores feed only on buds and flowers (McCall and Irwin 2006), move from leaves to flowers when plants are flowering (Smallegange et al. 2007, Agerbirk et al. 2010, Bandeili and Müller 2010, Lucas-Barbosa et al. 2013), or prefer flowers over leaves when given a choice (Rusman et al. chapter 6). Importantly, plant responses to herbivory are different between leaves and flowers, and between vegetative and flowering plants (Rusman et al. chapters 2, 4). Therefore, the outcome of interactions among herbivores that are feeding on flowers can be different than predicted by their feeding guild when feeding on leaves (Chrétien et al. 2018, Rusman et al. *unpublished*). We found that florivorous aphids prefer *B. nigra* plants infested with leaf-feeding aphids over uninfested plants. In contrast, florivorous aphids preferred uninfested plants over plants infested by a leaf- or a root-chewing herbivore. Florivorous caterpillars showed opposite responses compared to the florivorous aphids: the root-chewing herbivores had positive effects, the leaf-chewing herbivore no effect, and the leaf-feeding aphids had a negative effect on the preference of florivorous caterpillars. Surprisingly, florivorous caterpillars performed worse on plants infested with root-chewing herbivores compared with uninfested plants, whereas caterpillars performed best on plants infested with leaf-chewing herbivores and aphids. Performance of florivorous aphids was not affected by plant responses to herbivory on leaves or roots (Rusman et al. *unpublished*). Thus, variation in a combination of traits such as feeding guild and feeding site may be important for antagonist-antagonist interactions in plant-associated communities, and especially when herbivores feed on vegetative and floral tissues.

Trait variation in antagonist-mutualist interactions

Trait variation associated with herbivore feeding guild and feeding site also seems to be important for plant-mediated interactions between mutualists and antagonists. I have shown that the feeding guild of herbivores may be important for effects on visitation by different pollinator functional groups (Fig. 1) (Rusman et al. 2018 - chapter 3). For individual herbivore-pollinator interactions, variation in herbivore feeding guild did not explain the outcome of these interactions, but interactions were rather specific for herbivore and pollinator species (Fig. 2) (Rusman et al. 2019 - chapter 5). This was consistent with herbivore-induced changes in flower traits. Such changes in flower traits do not seem to depend on herbivore feeding guild, but rather are herbivore species-specific (Rusman et al. chapters 2, 5). Herbivore-induced changes in flower traits depend on the feeding site of the herbivore as well, with different changes in flower traits when herbivores are feeding on roots, leaves, or flowers (Farré-Armengol et al. 2015, Lucas-Barbosa et al. 2016, Rusman et al. chapter 2). Indeed, herbivore feeding position seems a good predictor for the outcome of some antagonist-mutualist interactions (Fig. 2) (Rusman et al. chapter 6). The importance of herbivore feeding position for plant-mediated herbivore-pollinator interactions was already hypothesized a decade ago (Kessler and Halitschke 2009), but has received limited attention so far. We found that different aphid species feeding on flowers had a consistent, positive effect on the visitation by two pollinating insects, whereas they did not when feeding on leaves (Rusman et al. chapter 6). Interestingly, the prediction by Kessler and Halitschke (2009) was that florivory would have especially negative effects on pollinator visitation. This prediction was mostly based on interspecific variation in feeding position among chewing florivores, whereas I used intraspecific trait variation among phloem-feeding aphids. There might be an interesting interaction effect between feeding guild and position that will determine the outcome of antagonist-mutualist interactions, and plant-mediated interactions in general.

It matters who is at the other end of the line

Mutualistic interactions may also provide important sources of trait variation that affect plant-mediated interactions between antagonists and mutualists. I have shown that pollinator functional groups vary in their responses to herbivore-induced plant responses in terms of attraction to herbivore-infested plants and various visitation parameters such as number of flowers visited and time spent per plant and flower. Functional groups of pollinators vary in their use of floral traits during flower visitation and this can lead to different responses to herbivore-induced changes in flower traits (Rusman et al. 2018 - chapter 3, 4). Naïve butterflies, diurnal moths, syrphid flies, and bumblebees prioritize colour/visual cues over scent for flower visitation, whereas nocturnal moths rely mostly on scent (Lunau 1992, Ômura and Honda 2005, Balkenius et al. 2006). Syrphid flies

may not be able to detect differences in the brightness of particular colours, in contrast to other pollinators (Lunau and Maier 1995, Sutherland et al. 1999). Unexperienced oligolectic solitary bees combine the use of colour and odours, but may prioritize the use of either one depending on the bee species (Burger et al. 2010, Dötterl et al. 2011, Milet-Pinheiro et al. 2012). Learning in pollinators is well established, especially for social pollinators like honeybees and bumblebees, but also for butterflies, whereas syrphid flies seem to have more limited learning capacities in relation to flower visitation (Lunau and Maier 1995, Riffell 2011). During learning, specific odour cues, colours, or shapes can be associated with flower rewards (Knauer and Schiestl 2015). Experience, therefore, temporarily modifies trait use by bumblebees and honeybees. For oligolectic solitary bees, experience only slightly modifies trait use, for example by bees relying on a larger number of volatile compounds (Burger et al. 2012) or increasing the strength of the response to visual cues (Dötterl et al. 2011, Milet-Pinheiro et al. 2012). Differential trait use, reward preference for nectar or pollen, and learning abilities likely explain differences in responses of pollinator functional groups to herbivore-induced changes in flower traits.

A pollinator's host-plant range can also provide an important source of trait variation for herbivore-pollinator interactions. Specialist and generalist pollinators differ in the use of flower traits during foraging. Specialist pollinators seem more sensitive to host-specific volatiles compared to generalist pollinators (Brandt et al. 2017). In contrast, specialist pollinators may be less sensitive to relatively high concentrations of secondary metabolites in nectar or pollen of their host plants, as opposed to generalist pollinators (Vanderplanck et al. 2018). Therefore, herbivore-induced changes in flower traits such as floral volatiles or secondary metabolites in nectar or pollen, likely have different effects on generalist and specialist pollinators. Future research needs to design experiments that explicitly compare generalist and specialist pollinators to elucidate how important variation in pollinator functional groups and host-plant range is for herbivore-pollinator interactions, or if these interactions are largely species-specific (Hoffmeister et al. 2016, Lucas-Barbosa 2016).

Consequences of herbivory for plant fitness

Elucidating broader patterns in indirect interactions, and identifying important traits in such interactions is not only important to explain ecological dynamics of communities, but also evolutionary dynamics. Evolution is driven by differences in the fitness of individuals, i.e. the contribution to the gene pool of the next generation. In the classic view of evolution, differences in fitness of individuals are driven by direct interactions with the environment. Indeed, when two species are taken out of community context, evolutionary changes driven by direct interactions can be demonstrated (Knauer and

Schiestl 2016, Gervasi and Schiestl 2017, Schiestl et al. 2018). Within a community context, indirect interactions also affect the fitness of community members, and thereby promote evolutionary change (Strauss et al. 2005, Turcotte et al. 2012, Guimarães Jr et al. 2017). Indeed, trait-mediated interactions between antagonists and mutualists may contribute to plant evolution (Ashman 2002, Irwin 2006, Ashman and Penet 2007, Poelman and Kessler 2016). I have shown that herbivory can have negative or positive effects on plant fitness, and the interplay between direct effects of herbivory and indirect effects *via* changes in pollinator visitation is likely important for plant fitness consequences (Fig. 1). Herbivore attack by specific herbivores on vegetative plants reduced the number of flowers, pollinator visitation, and seed production. On flowering plants, herbivore attack did not affect the number of flowers or pollinator visitation, but changed the composition of the pollinator community rather than the number of visits, and resulted in (over)compensation in terms of seed production (Rusman et al. 2018 - chapter 3, Rusman at al. chapter 4). Thus, the interplay between direct and indirect effects of herbivory is likely important for plant fitness consequences.

There is more to it than seeds alone

Before discussing direct and indirect effects of herbivory on plant fitness, it needs to be clear what plant fitness is. Most effects of herbivory on plant fitness are measured in number of seeds, but plant fitness constitutes more than seed production (Erb 2018). Fitness should be estimated by the relative number of offspring in the next generation (Primack and Kang 1989). For hermaphroditic plants, fitness constitutes a female and a male part. Female fitness is measured as the number of offspring in the next generation that came from the fertilized ovules of the plant (Primack and Kang 1989). This includes seed production, but also seed germination, survival and fecundity of the offspring. Herbivory can decrease, increase or have no effects on seed production (Rusman et al. 2018 - chapter 3). Seed germination and the often used proxy seed weight can increase or decrease as a result of herbivory (Agrawal 2001, Mueller et al. 2005, Gols et al. 2015, Rusman et al. chapter 4). Interestingly, herbivore attack on the mother plant can have positive effects on plant offspring growth and flower production (Steets and Ashman 2010).

In addition to plant female fitness, herbivory can affect plant male fitness. Male fitness is measured as the number of offspring in the next generation sired with pollen of that plant individual (Primack and Kang 1989). This includes pollen production, pollen germination, and seed siring ability. Herbivory can directly reduce male fitness by consuming pollen (Krupnick and Weis 1999) or reducing pollen production (Quesada et al. 1995, Rusman et al. 2019 - chapter 5). I found that herbivory by larvae of the root herbivore *Delia radicum* reduces pollen production of *B. nigra* to about 46,000 compared

to 49,000 for uninfested plants. Herbivory can reduce pollen germination (Rusman et al. *unpublished*), and pollen performance, e.g. competitive ability compared to pollen from uninfested plants (Quesada et al. 1995). Our results show that herbivory by larvae of the root herbivore *D. radicum* reduces pollen germination of *B. nigra* to 40% compared to 60% for uninfested plants and plants infested with *Liphaphis erysimi* aphids, and 50% for plant infested with *P. brassicae* (Rusman et al. *unpublished*). The extent to which measures such as pollen production or germination need to be reduced to have a significant impact on male fitness is unknown. Taken together, herbivory can affect multiple components of plant female and male fitness.

Measuring these multiple aspects of plant fitness is essential to determine the complete effects of herbivory on plant fitness. Negative effects of herbivory may be masked by only measuring female or male fitness. For example, foliar herbivory by *P. rapae* on *Raphanus raphanistrum* reduced male but not female fitness (Lehtilä and Strauss 1999). Resource allocation from pollen to ovule and/or seed production upon herbivory might ensure compensation in terms of female fitness at the cost of male fitness. Different aspects of female or male fitness may change differentially as a result of herbivory. For example, data in this thesis show that attack of *B. nigra* by *P. brassicae* and *Brevicoryne brassicae* reduced seed number, whereas attack by *B. brassicae* also reduced seed weight (Rusman et al. chapter 4). On *Sinapis arvensis* plants, attack by *P. brassicae* reduced seed number but increased seed weight and germination (Gols et al. 2015). Hence, herbivore attack may differently affect plant fitness parameters, partly due to resource allocation trade-offs. An alternative way of measuring effects of herbivory on plant fitness is performing selection experiments (Erb 2018). Selection experiments measure the outcome of herbivore-imposed selection and show that herbivory can result in trait changes and affect the genotypic composition of plant populations over multiple generations (Agrawal et al. 2012, Agrawal et al. 2013). For a mechanistic understanding of the outcome of herbivore-imposed selection, one needs to measure various fitness parameters nonetheless, and preferably more than seed production and germination (Waser et al. 2010, Agrawal et al. 2013, Campbell et al. 2017). Thus, although plant seed production can give an indication of potential fitness consequences, considering multiple aspects of plant fitness will improve our understanding of the impact of herbivory on plant fitness.

Direct effects of herbivory on plant fitness

Direct effects of herbivory on plant fitness are often negative (Gols et al. 2015, Lucas-Barbosa et al. 2017, Grass et al. 2018). Such direct negative effects can be due to the excessive consumption of the flowers or anthers, shifting resource allocation to defence instead of reproduction (Züst and Agrawal 2017), or lower productivity due to the loss of valuable photosynthetic tissue early in life (Boege et al. 2007, de Vries et al. 2018, 2019). For

example, pollen beetles can cause seed yield losses up to 80% in oilseed rape production fields, because of abscission of most buds due to high infestation loads (Hansen 2004). In stressful environments, direct negative effects of herbivory may be more prominent because of higher levels of resource limitation due to additional stresses like competition with other plants (Züst and Agrawal 2017). Plants can prevent negative effects of herbivory on plant fitness by compensation. Compensation occurs when damaged and undamaged plants have similar fitness. Despite the direct damage, annual plants seems to be well able to compensate for herbivore attack (Pashalidou et al. 2015, Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3, Rusman et al. chapter 4). Compensation can be a direct effect of herbivory *via* postponed or accelerated reproduction (Agrawal 2000a, Lucas-Barbosa et al. 2013, Pashalidou et al. 2013). Plants may phenologically separate resources to pre-herbivory and post-herbivory, and after herbivory has stopped allocate resources to reproduction (Agrawal 2000a). Alternatively, egg deposition by *P. brassicae* on *B. nigra* induces acceleration of flower and seed production (Lucas-Barbosa et al. 2013, Pashalidou et al. 2013). This ensures seed production before caterpillars (and other potential florivores) can reach and consume the flowers. Surprisingly, some direct effects of herbivory can cause overcompensation. Overcompensation occurs when damaged plants have a higher female and/or male fitness compared to undamaged plants. In the landmark studies by Paige and colleagues, it was illustrated how browsing by ungulates induced compensatory regrowth resulting in more flowers compared with unbrowsed plants, yielding higher seed and pollen production (Paige and Whitham 1987, Paige 1992, 1994, Gronemeyer et al. 1997). Overcompensation *via* postponed reproduction seems to have evolved under very specific conditions: a high and predictable risk of damage (Lennartsson et al. 1997, Agrawal 2000a). Such conditions are unlikely in most natural systems, where plants are under continuous attack and herbivore colonization is unpredictable. Thus, direct effects of herbivory on plant fitness can vary depending on the environment.

Indirect effects of herbivory on plant fitness

Herbivores can positively or negatively affect plant fitness indirectly *via* plant-mediated interactions. Negative effects on seed set may occur *via* herbivore-pollinator and herbivore-herbivore interactions. Herbivore-induced plant responses can result in fewer total pollinator visits or a lower visitation ratio of efficient pollinators, reducing the number of fertilized ovules and hence female fitness (Chautá et al. 2017). Negative effects on male fitness may occur *via* a reduction in pollen export following reduced pollinator visitation (Krupnick and Weis 1999). Herbivore-induced plant responses can result in the attraction of specialist herbivores (Poelman et al. 2010) with associated fitness costs (Wise and Rausher 2013). Plant-mediated interactions are also used by

plants to mitigate direct negative effects of herbivory and function as indirect defence by attracting natural enemies of herbivores, leading to compensation in plant seed production (Smallegange et al. 2008, Gols et al. 2015, Lucas-Barbosa et al. 2017). Positive effects on plant fitness may occur *via* herbivore-pollinator and herbivore-herbivore interactions. Herbivore-induced plant responses may result in higher reproductive output by attracting more pollinators or a higher ratio of efficient pollinators (Aizen and Harder 2007), or by inducing resistance against subsequently colonizing herbivores (Agrawal et al. 1999, Kessler and Baldwin 2004, McArt et al. 2013, Wise and Rausher 2013). I found overcompensation of *B. nigra* in response to root herbivory by three different root herbivores (Rusman et al. 2018 - chapter 3). Plants infested with root herbivores had a different pollinator community composition compared to uninfested plants. This community might provide better pollination of *B. nigra* because it contains a higher proportion of bumblebees compared to the community visiting uninfested plants, and bumblebees may be more efficient pollinators compared to honeybees or flies (Aizen and Harder 2007, Rader et al. 2009, Garibaldi et al. 2013). Alternative explanations are less likely; direct effects of root herbivores on plant fitness are often negative (van Dam 2009, Barber et al. 2015, Ghyselen et al. 2016), and I did not find changes in the colonization by important florivorous pollen beetles. More bumblebees could have been attracted by accelerated investment in flower and seed production (Lucas-Barbosa et al. 2013, Pashalidou et al. 2013) and/or changes in flower traits (Rusman et al. 2019 - chapter 5). Indeed, root herbivory has been shown to increase pollinator visitation by certain pollinator species in other studies as well (Poveda et al. 2003, 2005, Poveda et al. 2007, Barber et al. 2011). Hence, indirect effects of herbivory *via* plant-mediated interactions range from decreases to increases in plant fitness.

Direct and indirect effects of herbivory on plant fitness can be interdependent. The data presented in this thesis show that attack of *B. nigra* by *P. brassicae* and *B. brassicae* in the vegetative stage of the plant reduced the number of flowers, total pollinator visitation, and seed production (Rusman et al. chapter 4). Attack of *B. nigra* by *L. erysimi* in the bud stage (when the first flower stalk with buds rises above the canopy) or *D. radicum* in the flowering stage seems to reduce the number of flowers, but not the number of total pollinator visits, and did not reduce seed production. Attack of *B. nigra* by the sawfly *Athalia rosae* or the nematode *Heterodera schachtii* in the bud stage, respectively, reduced or increased the number of total pollinator visits, whereas neither affected seed production. Only a combination of a reduction in the number of flowers and in pollinator visitation as a result of herbivory reduced plant seed production (Rusman et al. chapter 4). Taken together, both direct and indirect effects of herbivory are likely important for plant fitness consequences.

Eco-evolutionary dynamics driven by plastic flowers

Indirect interactions are highly integrated in ecological and evolutionary dynamics of multispecies communities. Effects of indirect interactions on community organization and trait evolution generate eco-evolutionary feedback because indirect interactions are often trait-mediated, and trait evolution shapes indirect interactions (Walsh 2013). Eco-evolutionary dynamics driven by indirect interactions are known for antagonist and mutualist networks. Trait variation in feeding behaviour can affect other community members *via* indirect antagonistic interactions (Johnson et al. 2009, Farkas et al. 2013, Matthews et al. 2016, Ohgushi 2016), and in turn, changes in the community affect trait evolution by changing selection regimes (Siepielski and Benkman 2004, Agrawal et al. 2012, Bassar et al. 2012). In mutualist networks, indirect interactions affect network structure and trait evolution (Guimarães Jr et al. 2011, Rodríguez-Echeverría and Traveset 2015, Rodríguez-Echeverría et al. 2016, Guimarães Jr et al. 2017), which in turn shifts interaction networks (Santamaría and Rodríguez-Gironés 2007, Nuismer et al. 2013). Eco-evolutionary dynamics have mostly been investigated separately for antagonist and mutualist networks (Fontaine et al. 2011). In plant-associated communities, antagonists and mutualists co-occur, and eco-evolutionary dynamics likely involve both antagonists and mutualists.

Herbivore - flower-visitor interactions have the potential to drive eco-evolutionary dynamics in multiple ways. Herbivore-induced changes in flower traits can alter pollinator visitation (Soper Gorden and Adler 2016, Rusman et al. chapters 4, 5) and pollinator community composition (Steets et al. 2006, Hoffmeister et al. 2016, Rusman et al. 2018 - chapter 3), but also visitation by antagonistic nectar thieves, robbers and florivores (Soper Gorden and Adler 2016, Ikemoto et al. 2017, Soper Gorden and Adler 2018, Rusman et al. chapter 4), and florivore community composition (Stam et al. 2018). Changes in interactions with flower visitors impose selection on flower traits and plant defence (Johnson et al. 2015, Lucas-Barbosa 2016, Poelman and Kessler 2016), which can lead to rapid evolution of plant traits (Schiestl and Johnson 2013, Gervasi and Schiestl 2017). Evolutionary change in plant traits subsequently feeds back to affect interactions with flower visitors, both mutualists and antagonists, and the initially attacking herbivore (McCall and Irwin 2006, Strauss and Whittall 2006, McCall et al. 2013), completing the eco-evolutionary feedback loop. Changes in the interactions between plants, herbivores and flower visitors can drive evolutionary changes in the herbivores and flower visitors, which subsequently affect the interactions of these community members with the plant, adding another eco-evolutionary feedback loop. This process will repeat itself. Although we have no examples of this process yet, a similar eco-evolutionary feedback loop through inducible plant responses seems to affect the evolution of feeding preference of

leaf beetles on willow and its associated arthropod community including herbivores and predators (Utsumi et al. 2013, Utsumi and Shefferson 2015). Therefore, it seems likely that antagonist-mutualist interactions can drive eco-evolutionary dynamics through inducible plant responses.

Conclusion

In this thesis I have shown that for herbivore - flower-visitor interactions, trait variation among the different interactors is an important determinant of the effects of these indirect interactions: trait variation in either herbivores, plants, or flower visitors affects flower-visitor community dynamics and has associated plant fitness consequences. The combination of herbivore feeding guild and feeding site may partially determine the direction and strength of herbivore-pollinator and herbivore-herbivore interactions. Herbivore-induced changes in flower traits are to a large extent herbivore species-specific and thus even herbivore identity is important for herbivore - flower-visitor interactions. Ontogenetic variation in plant traits increases specificity even further, and the indirect interaction web between herbivores and flower visitors seems dynamic over plant ontogeny, with consequences for plant fitness. Herbivore attack by specific herbivores on vegetative plants reduced the number of flowers, pollinator visitation, and seed production. On flowering *B. nigra* plants, herbivore attack did neither affect the number of flowers nor change the overall numbers of pollinators visiting flowers, but changed the composition of the pollinator community, and resulted in (over)compensation in seed production. I have shown that herbivory can have negative or positive effects on plant fitness, and the interplay between direct effects of herbivory and indirect effects *via* changes in pollinator visitation is likely important for the reproductive output of a plant. Because herbivory affects multiple aspects of plant female and male fitness, measuring seed production may not provide a complete understanding of plant evolution driven by herbivores. Nonetheless, positive and negative effects of herbivory on plant fitness have shown that herbivores may play significant roles as agents of selection on flower traits by affecting plant interactions with flower visitors. Therefore, plant-mediated interactions, including herbivore - flower-visitor interactions, are likely reflected in natural selection on plant growth-defence-reproduction strategies.

Most communities are composed of both antagonists and mutualists. The chapters of this thesis show that antagonist-mutualist interactions can have considerable impact on the dynamics of a mutualist network, with associated fitness consequences for plants. Recent synthesis argues that antagonist-mutualist interactions can have profound consequences for mutualist fitness (Jacobsen and Raguso 2018). In addition, mutualist-antagonist interactions can also impact antagonist network dynamics and antagonist fitness (Pineda

et al. 2010, Soper Gorden and Adler 2018). To understand ecological and evolutionary dynamics of communities, we need to consider antagonist and mutualist networks as integrated components of the full community, rather than separate networks. An important knowledge gap regarding mutualist-antagonist interactions is the importance of trait variation as determinant of the outcome of eco-evolutionary dynamics. The results of my thesis show that both inter- and intraspecific variation in different traits related to the feeding behaviour of antagonists is important in determining the linkage between antagonist and mutualist networks. Thus, trait variation in antagonists can be a profound driver of eco-evolutionary dynamics in communities by affecting mutualistic community members.

Future perspectives

In the field, plants are colonized by a multitude of herbivores over the growing season. Different herbivore species can arrive at approximately the same time or sequentially. At any given time, plants can harbour a mix of herbivore species. Currently, we do not know how multiple herbivores - simultaneously or in sequence - differentially affect visitation by flower visitors and whether these multi-species interactions have plant fitness consequences (but see Stam et al. 2018). Moreover, investigating the effect of plant exposure to variation in herbivore diversity and herbivore functional traits, such as feeding guild and feeding site, will give additional insights into the importance of biodiversity and trait composition in community assembly. Adopting a mechanistic point of view raises questions about how simultaneous or sequential attack by multiple herbivores will change flower traits, and if such changes will be additive or non-additive. Combining flower-visitor observations with flower-trait measurements *in the field* will shed light on the question whether herbivore diversity increases specificity of induction of flower traits and effects on flower visitors. It is to date poorly understood whether and how these multi-species interactions impact on plant fitness.

To increase our understanding of effects of herbivore diversity and multi-species interactions on plant fitness we need to partition the direct and indirect effects, and elucidate the contribution of various components of plant-associated communities. Direct effects of herbivory on plant fitness can be revealed by comparing the fitness of plants experimentally infested with herbivores and uninfested plants, and including or excluding the natural plant-associated community. By using mesh cages or applying pesticides, and growing plants in sterilized soil the natural plant-associated community can be largely excluded. To expose the indirect effects of herbivory on plant fitness, the plant-associated community on herbivore-infested and uninfested plants should be monitored. The contribution of different networks to plant fitness can be disassembled by Structural Equation Modelling (Herrera et al. 2002, Cariveau et al. 2004). Combining these measures will not only deepen our understanding of plant fitness consequences

of herbivore attack, but also how effects of herbivore attack will echo through the plant-associated community. Including trait measurements will allow researchers to infer agents of selection on plant defence and reproductive traits. This will increase our understanding of the importance of indirect interactions for natural selection, which is essential for understanding plant evolution.

Plant-insect communities provide excellent systems to gain insights in eco-evolutionary dynamics of complex multispecies communities with antagonists and mutualists. The results of my thesis show that both inter- and intraspecific variation in different traits related to the feeding behaviour of antagonists is important in determining the linkage between antagonist and mutualist networks. A next step will be to show that changes in mutualist community assembly feed back to affect antagonist fitness, closing the eco-evolutionary feedback loop.

In conclusion, this thesis makes an important contribution to our understanding of how species traits can influence ecological network structure. The demonstrated importance of trait variation in plant-mediated herbivore - flower-visitor interactions for flower visitor community dynamics and associated plant fitness consequences will stimulate further research on eco-evolutionary dynamics in multispecies communities with antagonists and mutualists.

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Summary

Understanding the interplay between ecology and evolution in complex multispecies communities is a major challenge for ecologists. Plant-insect systems provide excellent models to study ecological and evolutionary dynamics in complex multispecies communities because plants are under selection to maximize fitness in a community context including antagonistic and mutualistic insects. Most plant species are flowering plants, and to maximize their contribution to the next generation they need to deal with antagonistic herbivores while also engaging in interactions with mutualistic pollinators. Plants respond to attack by herbivores with phenotypic changes to repel or kill the attackers. Plant phenotypic changes do not only affect the attacker, but alter interactions with other community members as well. For instance, when plants are flowering, antagonist attack to leaves or roots can induce phenotypic changes in flowers. As a result, herbivore attack can alter interactions with pollinators and flower feeders. Such plant-mediated herbivore - flower-visitor interactions may be herbivore-specific. It is well known that leaf-chewing and sap-feeding herbivores induce different signal-transduction pathways in leaves that cascade into different plant phenotypic responses. Although very little is known about specificity in flower trait expression after leaf herbivory, these specific plant responses to herbivory may extend to flower trait expression. Both antagonists and mutualists influence the reproductive output of the plant, and altered plant interactions with antagonists and mutualists due to herbivory can have consequences for plant reproduction. Knowledge on the importance of indirect interactions for the assembly of flower-visitor networks on plants and associated consequences for plant fitness is limited to date.

The aim of this thesis project was to investigate how attack by a range of herbivore species affects plant interactions with mutualistic and antagonistic flower visitors and whether these interactions have consequences for plant fitness. I was especially interested in specificity of plant-mediated herbivore - flower-visitor interactions. The study used the Black mustard (*Brassica nigra*) plant and ten different herbivore species with different feeding behaviours: some choose to feed among their favourite tissues, others are highly specialized and engage in intimate and manipulative feeding relations with the plant, whereas a few take bites or sips from different plant parts. Black mustard is an annual plant species which relies on insect pollinators for reproduction. The large fragrant inflorescences contain hundreds of small yellow flowers which attract various generalist pollinators, but also specialist florivores such as pollen beetles, *Meligethes* spp.

Chapter 2 addresses the current knowledge on flower plasticity in response to herbivory and places flower plasticity in a community context. The chapter reviews the extent to which herbivore-induced plant responses affect multiple flower traits, and the molecular mechanisms underlying floral plasticity. Herbivore-induced changes in flower traits seem to be to a large extent herbivore species-specific, mediated by plant-tissue-specific and plant-ontogeny-specific gene expression and regulatory components. The herbivore-

induced changes in floral traits affect pairwise interactions with flower visitors and may seem adaptive in some cases, such as increased resistance to florivores, while maladaptive in others, such as the reduced visitation by pollinators. To understand the adaptive value of flower plasticity with such contrasting differences on pairwise interactions, it is important to adopt a community perspective. Indeed, herbivore-induced changes in flower traits have flower-community wide consequences and result in a highly connected web of interactions between antagonistic and mutualistic organisms. Still, specificity in plant-mediated herbivore - flower-visitor interactions as well as its consequences for flower-visitor community assembly and plant reproduction is poorly understood to date.

The main goal of the first field experiment was to evaluate specificity of plant responses to different herbivore species and how these plant responses affect flower visitors and plant fitness (**Chapter 3**)**.** Specificity of plant responses induced by herbivores is hypothesized to underlie patterns in plant-mediated herbivore - flower-visitor interactions. Herbivores with similar feeding guilds and that feed on similar plant tissues induce more similar plant responses than herbivores that differ in feeding guild or feeding site. In the field, *B. nigra* plants were exposed to a broad range of herbivores to investigate the effects of herbivore-induced plant responses on pollinator behaviour and whether the direction of interactions was predicted by the feeding mode (chewing and sap-feeding) and feeding site (above- and belowground) of the herbivores. Finally, consequences for plant fitness were investigated. The results show that attack of *B. nigra* by a range of different herbivores influenced plant interactions with mutualistic pollinators and an antagonistic florivore, the pollen beetle *Meligethes aeneus*. Plant exposure to herbivores affected pollinator community composition, whereas overall pollinator attraction was maintained. Pollinator community composition of uninfested plants differed from that of chewing and root-herbivore-infested plants. Main responders in the pollinator community to changes induced by herbivory were syrphid flies, bumblebees, and solitary bees. The changes in pollinator community composition and syrphid fly visitation may explain the observed increase in seed set of root herbivore-infested plants. The results presented in chapter 3 show specificity of plant-mediated herbivore - flower-visitor interactions that arises from herbivore- and functional-group-specific-induced plant responses as well as differential responses of various flower visitors to herbivore-induced plants. Although *B. nigra* plants maintained reproductive output despite these indirect interactions between antagonists and mutualists, feeding by herbivores from specific functional groups may enhance reproductive output. These results indicate that indirect plant-mediated interactions are likely reflected in natural selection on plant growth, defence, and reproductive strategies.

To maximize reproduction, plants are considered to display ontogenetic variation in growth, defence, and reproduction. Returning to the field, in the study presented in **Chapter 4**, my aim was to investigate whether herbivore - flower-visitor interactions

and associated plant fitness consequences are different when timing of herbivore attack varies over plant ontogeny. It is known that the costs of herbivory vary over plant ontogeny. Moreover, plant responses to herbivory also vary over plant ontogeny, and these responses extend to effects on flower abundance and other traits that influence flower visitors. Therefore, I expected ontogenetic variation in plant-mediated herbivore - flower-visitor interactions and consequences for plant reproductive output. In a common garden experiment, I examined whether exposure to various herbivore species on different plant ontogenetic stages (vegetative, bud, or flowering stage) affects plant flowering traits, interactions with flower visitors, and results in fitness consequences for the plant. The data presented in chapter 4 show that plant interactions with flower visitors such as pollinators and pollen beetles (*Meligethes* spp.) are affected by plant exposure to herbivores in all three plant ontogenetic stages tested. The outcome of herbivore - flower-visitor interactions was specific for the combination of herbivore species and plant ontogeny. Herbivory resulted in either positive or negative effects on pollinator visitation (attraction, number of flowers visited, time spent per visit and per flower) and generally reduced pollen beetle numbers compared to their abundance on undamaged plants. Effects of herbivory on flower visitors were most prominent when plants were exposed to herbivores in the vegetative or bud stage. Plant flowering traits and reproductive output were especially affected when plants in the vegetative stage were exposed to the herbivores. Especially exposure of vegetative plants to *Brevicoryne brassicae* aphids and *Pieris brassicae* caterpillars negatively affected inflorescence number and seed production. The indirect interaction web between herbivores and flower visitors appears dynamic and variable over plant ontogeny. This study shows that consequences of herbivory for plant reproductive output are strongest when plants are attacked by herbivores early in life. Such differences in selection pressures imposed by herbivores to specific plant ontogenetic stages likely drive the evolution of distinct ontogenetic trajectories in growth-defence-reproduction strategies and include indirect interactions between herbivores and pollinators.

In **Chapter 5**, I explored the underlying mechanisms of specificity in herbivore - flower-visitor interactions. I expected flower plasticity to follow patterns of specificity in inducible defences known for foliar plant responses, because flower and defence traits are physiologically linked. Inducible defences are regulated by phytohormones, which are involved in defence as well as in reproduction. Chewing herbivores, for instance, mainly induce the jasmonic acid (JA) pathway, whereas sap-feeding herbivores usually suppress the JA pathway and/or induce the salicylic acid (SA) pathway, resulting in specific plant responses to different herbivore species. In addition, reproduction and defence may be linked by shared genetic or biochemical pathways, *via* shared resources, or *via* functional responses, where flower traits are involved in defence as well. To elucidate if specific plant responses to herbivory may extend to flower-trait expression, flowering *B. nigra*

plants were exposed to one of six insect herbivore species and changes in a number of flower traits (flower abundance, morphology, colour, volatile emission, nectar quantity, and pollen quantity and size) and the behaviour of two pollinating insects were recorded. I show that herbivory can affect multiple flower traits and pollinator behaviour. The most plastic floral traits were flower morphology, colour, the composition of the volatile blend, and nectar production. Herbivore-induced changes in flower traits resulted in positive, negative or neutral effects on pollinator behaviour. Effects on flower traits and pollinator behaviour are herbivore species-specific. Flowers show extensive plasticity in response to herbivores, with contrasting effects on pollinators.

Specificity of herbivore-induced changes in flower traits might go beyond interspecific variation in herbivore feeding behaviour. When plants start flowering, some herbivores move upwards to feed on the flowers, while others remain feeding on leaves. Intraspecific variation in herbivore choice for leaves or flowers may lead to differences in inducible plant responses including flower traits and may affect pollinator visitation. In **Chapter 6**, I use manipulative experiments to explore whether herbivore choice for a given feeding site determines the outcome of plant-mediated herbivore-pollinator interactions. Three herbivore species were each placed on either leaves or flowers of flowering *B. nigra* plants and the responses of two pollinator species visiting flowers were recorded. I measured the preference of the herbivores for foliar and floral tissues and their performance on both tissues. The results show that variation in herbivore feeding site has profound impact on the outcome of herbivore-pollinator interactions. Herbivores feeding on flowers had consistent positive effects on pollinator visitation, whereas herbivores feeding on leaves did not. Herbivores themselves preferred to feed on flowers, and mostly performed best on flowers. Hence, the choice of the feeding site by the adult herbivores maximizes the species performance and had profound impact on the outcome of indirect interactions between herbivores and pollinators. Placed in a broad eco-evolutionary framework, the results of chapter 6 suggest that the evolution of antagonist feeding behaviour might affect community assembly *via* trait-mediated interactions. Therefore, antagonist-mutualist interactions may drive eco-evolutionary dynamics *via* inducible plant responses.

The data presented in this thesis contribute to our understanding of the complex ecological and evolutionary dynamics in multispecies communities with antagonists and mutualists, and this is discussed in **Chapter 7**. I have shown that for herbivore - flower-visitor interactions, trait variation among the different interactors is an important determinant of the outcome of these indirect interactions: trait variation in either herbivores, plants, or flower visitors affects flower-visitor community dynamics and has associated plant fitness consequences. The combination of herbivore feeding guild and feeding site may partially determine the direction and strength of herbivore-pollinator and herbivore-herbivore interactions. Herbivore-induced changes in flower traits are to a large extent herbivore species-specific and thus herbivore identity is important and can

influence the outcome of herbivore - flower-visitor interactions. Ontogenetic variation in plant traits increases specificity even further, and the indirect interaction web between herbivores and flower visitors seems dynamic over plant ontogeny, with consequences for plant fitness. Herbivory can have negative or positive effects on various plant fitness components, including female and male fitness. The interplay between direct effects of herbivory and indirect effects *via* changes in pollinator visitation is likely important for plant fitness and should be included in frameworks on plant trait evolution.

Samenvatting

Begrip van de wisselwerking tussen ecologie en evolutie in soortrijke gemeenschappen is een van de grote uitdagingen voor ecologen. Plant-insect systemen zijn uitstekende modellen om ecologische en evolutionaire dynamiek in soortrijke gemeenschappen te bestuderen, omdat planten onder natuurlijke selectiedruk staan om voortplanting te maximaliseren terwijl zij worden omgeven door een gemeenschap van schadelijke en nuttige insecten. Het merendeel van de plantensoorten bestaat uit bloeiende planten, en om hun bijdrage aan de volgende generatie (fitness) te maximaliseren moeten zij zich verdedigen tegen schadelijke plantenetende insecten (herbivoren) maar ook bestuivende insecten aantrekken. Planten reageren op aanvallen van herbivoren met veranderingen in hun fenotype zoals bijvoorbeeld de productie van giftige stoffen om zo de aanvaller te verjagen of te doden. Zulke veranderingen beïnvloeden niet alleen de belager, maar ook interacties tussen de plant en andere leden van de insectengemeenschap. Bijvoorbeeld, wanneer planten bloeien kan een aanval door herbivoren leiden tot veranderingen in bloemkenmerken, zoals veranderingen in bloemgeur. Dit kan vervolgens resulteren in een verminderde aantrekking van bloemeters, maar ook bestuivers. Zulke indirecte interacties tussen herbivoren en bloembezoekende insecten die het gevolg zijn van veranderingen in bloemkenmerken als gevolg van vraat zijn mogelijk afhankelijk van de soort herbivoor die de plant aanvalt. Het is bekend dat blad-knagende en sap-zuigende herbivoren verschillende fysiologische signaleringsroutes in de plant activeren, die resulteren in verschillende veranderingen in het uiterlijk van de plant. Hoewel er niet veel bekend is over de specificiteit van herbivoor-geactiveerde veranderingen in bloemkenmerken, zouden specifieke veranderingen in de plant in reactie op belagers kunnen leiden tot specifieke veranderingen in bloemkenmerken. Zowel schadelijke als nuttige insecten beïnvloeden de voortplanting van de plant, en veranderingen in de interacties tussen de plant en schadelijke of nuttige insecten als gevolg van vraatschade kan gevolgen hebben voor plantenfitness. Kennis van het belang van zulke indirecte interacties voor de samenstelling van de leefgemeenschap van bloembezoekers en bijkomende gevolgen voor plantenfitness is beperkt.

Het doel van dit onderzoeksproject was om te bepalen hoe een veelvoud aan verschillende herbivore insecten interacties tussen de plant en zowel schadelijke als nuttige bloembezoekers kan beïnvloeden, en mogelijke gevolgen voor plantenfitness. Ik was vooral geïnteresseerd in de specificiteit van deze herbivoor-bloembezoeker interacties. De studie heeft gebruik gemaakt van zwarte mosterd (*Brassica nigra*) planten, en tien verschillende soorten herbivoren die verschillen in hun voedingsgedrag: sommige soorten kiezen tussen hun favoriete weefsels, andere zijn zeer gespecialiseerd en gaan een intieme en manipulatieve relatie aan met de plant, weer andere nemen kleine hapjes of slurpjes van verschillende plantdelen. Zwarte mosterd is een eenjarige plant die afhankelijk is van bestuiving voor de voortplanting. De grote, geurige bloeiwijzen bestaan uit honderden kleine gele bloemen die aantrekkelijk zijn voor verschillende generalistische bestuivers,

maar ook specialistische bloemeters zoals stuifmeelkevers (*Meligethes* soorten).

Hoofdstuk 2 behandelt de huidige kennis over de plasticiteit van bloemen als reactie op vraatschade en plaatst bloemplasticiteit in het netwerk van interacties van de de bloem omringende levensgemeenschap. Het hoofdstuk bespreekt de mate waarin verschillende bloemkenmerken worden beïnvloed doordat planten reageren op herbivoren en wat de onderliggende moleculaire mechanismen zijn. Herbivoor-geactiveerde veranderingen in bloemkenmerken lijken voor een groot deel specifiek te zijn voor de soort herbivoor die de plant aanvalt doordat de expressie van plantengenen en belangrijke regulatiecomponenten van plantenreacties verschillen tussen plantenweefsels, en de ontwikkelingsfase van de plant. Herbivoor-geactiveerde veranderingen in bloemkenmerken beïnvloeden één-op-één interacties met bloembezoekers en lijken daardoor in sommige gevallen voordelig voor de plant, zoals een hogere resistentie tegen bloemeters, maar in andere gevallen nadelig, zoals een vermindering in bezoek door bestuivers. Om de adaptieve waarde van bloemplasticiteit te begrijpen, is het belangrijk om bloemplasticiteit in de context van een levensgemeenschap te plaatsen. Herbivoor-geactiveerde veranderingen in bloemkenmerken kunnen gevolgen hebben voor de gehele gemeenschap van bloem-gerelateerde organismen, en resulteren daardoor in een nauw verbonden netwerk van interacties tussen schadelijke en nuttige organismen. Ondanks dit gegeven is er nog weinig bekend over de specificiteit van herbivoor-bloembezoeker interacties, de gevolgen voor de opbouw en structuur van levensgemeenschappen van bloembezoekers, en effecten op plantenfitness.

Het hoofddoel van het eerste veldexperiment was om de mate van specificiteit van plantenreacties op verschillende soorten herbivoren te evalueren, en hoe deze plantenreacties effecten hebben op bloembezoekers en plantenreproductie (**Hoofdstuk 3**). De hypothese is dat specificiteit van plantenreacties geactiveerd door herbivoren de uitkomst van herbivoor-bloembezoeker interacties kan verklaren. Herbivoren die op dezelfde manier de plant aanvallen (knagende insecten zoals rupsen en kevers; sap-zuigende insecten zoals bladluizen) en die zich voeden met dezelfde plantenweefsels activeren meer op elkaar lijkende plantenreacties dan herbivoren die op verschillende manieren de plant aanvallen of die zich voeden met verschillende plantenweefsels. In het veld werden zwarte mosterd planten blootgesteld aan één van tien verschillende soorten herbivoren om zo de effecten van herbivoor-geactiveerde plantenreacties op het gedrag van bestuivers te onderzoeken, en te bepalen of de uitkomst kan worden voorspeld aan de hand van de manier van voeden (knagend of sap-zuigend) of de plek van voeden (boven- of ondergronds) van de herbivoren. Ook hebben we de gevolgen voor de voortplanting van de plant onderzocht. De resultaten laten zien dat vraatschade door verschillende herbivoren de interacties tussen zwarte mosterd en bestuivers als ook een schadelijke bloemeter, de stuifmeelkever *Meligethes aeneus*, beïnvloedt. Blootstelling van de plant aan herbivoren had invloed op de samenstelling van de bestuivergemeenschap,

terwijl de totale hoeveelheid bezoeken van bestuivers hetzelfde bleef. De samenstelling van de bestuivergemeenschap van planten die niet waren blootgesteld aan herbivoren verschilde van planten die waren blootgesteld aan bovengrond-knagende herbivoren en ondergrondse herbivoren. Vooral zweefvliegen, hommels, en solitaire bijen reageerden op plantenreacties op herbivoren. De veranderingen in de bestuivergemeenschap en bezoeken door zweefvliegen kunnen mogelijk de hogere zaadproductie verklaren van planten die zijn blootgesteld aan ondergrondse herbivoren. De resultaten in hoofdstuk 3 laten zien dat de specificiteit van herbivoor-bloembezoeker interacties verklaard kan worden aan de hand van zowel soort herbivoor- als functionele groep- (knagende, sap-zuigende, of wortel-etende) specifieke plantenreacties en verschillende reacties van bloembezoekers op planten die zijn blootgesteld aan herbivoren. Zwarte mosterd planten kunnen de zaadproductie op peil houden ondanks indirecte interacties tussen schadelijke en nuttige organismen, en vraatschade door specifieke herbivoren kan zelfs de voortplanting ten goede komen. Deze resultaten suggereren dat indirecte interacties mogelijk selectiedruk uitoefenen op plantenstrategieën in groei, verdediging, en voortplanting.

Om de voortplanting te maximaliseren vertonen planten verschillende strategieën met betrekking tot groei, verdediging, en voortplanting afhankelijk van de ontwikkelingsfase (vegetatief, in knop, in bloei) waarin de plant zich bevindt. Terug in het veld met de studie gepresenteerd in **Hoofdstuk 4** was het mijn doel om te onderzoeken of herbivoor-bloembezoeker interacties en daaruit voorvloeiende gevolgen voor plantenreproductie verschillen afhankelijk van de timing van vraatschade met plantenontwikkeling. Het is bekend dat de kosten van vraatschade voor de plant verschillen afhankelijk van de ontwikkelingsfase waarin de plant zich bevindt. Ook reacties van planten op vraatschade verschillen afhankelijk van de ontwikkelingsfase, en dit heeft effect op het aantal bloemen en andere bloemkenmerken die bloembezoekers aantrekken. Zodoende verwachtte ik variatie in herbivoor-bloembezoeker interacties en gevolgen voor plantenreproductie afhankelijk van de ontwikkelingsfase waarin de plant belaagd wordt. In een veldexperiment heb ik onderzocht of de blootstelling van planten in verschillende ontwikkelingsfasen (vegetatief, in knop, in bloei) en aan verschillende soorten herbivoren een effect had op bloeieigenschappen (aantal bloemhoofden, begin en duur van bloei) van de plant. Ook heb ik effecten op interacties met bloembezoekers en de gevolgen voor plant reproductie onderzocht. De resultaten gepresenteerd in hoofdstuk 4 laten zien dat interacties van planten met bloembezoekers zoals bestuivers en stuifmeelkevers (*Meligethes* soorten) worden beïnvloed door blootstelling aan herbivoren in alle drie de ontwikkelingsfases die zijn getest. De uitkomst van herbivoor-bloembezoeker interacties was specifiek voor de combinatie van de soort herbivoor en de fase van plantenontwikkeling. Vraatschade resulteerde in positieve of negatieve effecten op bestuiverbezoeken (aantrekking, hoeveelheid bloemen bezocht, tijd gespendeerd per bezoek aan een plant en per bloem).

Over het algemeen verlaagde vraatschade de hoeveelheid stuifmeelkevers op planten in vergelijking met de hoeveelheid op intacte planten. De effecten van vraatschade op bloembezoekers waren het sterkst wanneer vraatschade plaats vond in de vegetatieve of knopfase van de plant. Bloeieigenschappen en de voortplanting van planten werden vooral beïnvloed door vraatschade tijdens de vegetatieve fase. Vooral vraatschade door de melige koolluis (*Brevicoryne brassicae*) en rupsen van het grote koolwitje (*Pieris brassicae*) hadden een negatief effect op de hoeveelheid bloemhoofden en zaadproductie. Het netwerk van interacties tussen herbivoren en bloembezoekers lijkt dynamisch en variabel gedurende de ontwikkeling van de plant. Deze studie leert ons dat de gevolgen van vraatschade voor plantenfitness het sterkst zijn als planten vroeg in hun leven worden aangevallen door herbivoren. De verschillen in selectiedruk van herbivoren tijdens specifieke ontwikkelingsfases van de plant zijn waarschijnlijk belangrijk voor de evolutie van verschillende strategieën in groei-verdediging-reproductie, en deze verschillen in selectiedruk kunnen worden beïnvloed door indirecte interacties tussen herbivoren en bloembezoekers.

In **Hoofdstuk 5** onderzoek ik de onderliggende mechanismen van specificiteit in herbivoor-bloembezoeker interacties. Ik verwacht dat specificiteit van herbivoor-geactiveerde veranderingen in bloemkenmerken weerspiegeld wordt in de specificiteit van herbivoor-geactiveerde verdedigingseigenschappen bekend van plantenreacties gemeten in bladweefsel, omdat bloemkenmerken en verdedigingseigenschappen fysiologische verbonden zijn. Herbivoor-geactiveerde verdedigingseigenschappen worden gereguleerd door plantenhormonen, welke zowel bij de verdediging als voortplanting betrokken zijn. Knagende herbivoren activeren bijvoorbeeld vooral het plantenhormoon jasmonzuur, terwijl sap-zuigende insecten vaak jasmonzuur onderdrukken en een ander plantenhormoon activeren: salicylzuur. Hierdoor ontstaan specifieke plantenreacties op verschillende belagers. Verder kunnen voortplanting en verdediging fysiologisch verbonden zijn via gedeelde genetische of biochemische routes, via gedeelde bouwstoffen zoals koolhydraten of stikstof, of via gedeelde functies waarbij bloemkenmerken ook verdedigende functies hebben. Om te onderzoeken of specifieke reacties van planten op belagers gevolgen hebben voor de expressie van bloemkenmerken werden bloeiende zwarte mosterd planten blootgesteld aan één van zes soorten herbivore insecten. Vervolgens werden veranderingen in een aantal bloemkenmerken (bloemaantallen, morfologie, kleur, geur, nectarproductie, en stuifmeel hoeveelheid en grootte) en het gedrag van twee bestuivers gemeten. De resultaten laten zien dat vraatschade meerdere bloemkenmerken en het gedrag van bestuivers beïnvloedt. De meest plastische bloemkenmerken waren morfologie, kleur, geur, en nectarproductie van bloemen. Herbivoor-geactiveerde veranderingen in bloemkenmerken resulteerden in positieve, negatieve, of neutrale effecten op het gedrag van bestuivers. De effecten op bloemkenmerken en het gedrag van bestuivers waren specifiek voor de soort herbivoor

die van de plant at. Bloemen laten omvangrijke plasticiteit zien in reactie op belagers, met contrasterende gevolgen voor het gedrag van bestuivers.

Herbivoor-geactiveerde veranderingen in bloemkenmerken kunnen specifieker zijn dan variatie in voedingsgedrag tussen soorten. Wanneer planten beginnen te bloeien, klimmen sommige herbivoren omhoog om zich tegoed te doen aan de bloemen, terwijl andere van de bladeren blijven eten. Variatie binnen soorten in de keuze om zich te voeden met bladeren of bloemen kan leiden tot verschillende reacties van de plant inclusief bloemkenmerken, met mogelijke gevolgen voor de aantrekking van bestuivers. In **Hoofdstuk 6** gebruik ik een manipulatief experiment om te onderzoeken of de keuze van herbivoren voor een bepaalde voedingsplek de uitkomst van herbivoor-bestuiver interacties bepaalt. Drie soorten herbivoren werden op de bladeren of bloemen geplaatst van bloeiende zwarte mosterd planten, en de reacties van twee soorten bestuivers werd getest. Ook heb ik de voorkeur van de herbivoren voor, en de groei op, bladeren en bloemen getest. De resultaten laten zien dat variatie in voedingsplek van herbivoren een grote invloed heeft op de uitkomst van herbivoor-bestuiver interacties. Belagers op bloemen hadden een consistent positief effect op bezoek door bestuivers, terwijl belagers op bladeren dat niet hadden. De herbivoren zelf hadden een voorkeur voor bloemen, en de meesten groeiden het best op bloemen. De keuze voor een voedselplek door volwassen herbivoren zorgt voor maximale groei en heeft een sterke invloed op de uitkomst van indirecte interacties tussen herbivoren en bestuivers. Als we de resultaten in een breder eco-evolutionair (wisselwerking tussen ecologie en evolutie) kader plaatsen suggereren de onderzoeksgegevens van hoofdstuk 6 dat de evolutie van het voedingsgedrag van belagers de netwerkstructuur van levensgemeenschappen kan beïnvloeden. Daardoor kunnen interacties tussen schadelijke en nuttige organismen eco-evolutionaire dynamiek beïnvloeden via geactiveerde plantenreacties.

De onderzoeksresultaten gepresenteerd in dit proefschrift dragen bij aan ons begrip van de ingewikkelde ecologische en evolutionaire dynamiek in soortenrijke gemeenschappen met belagers en nuttige organismen, en dit wordt bediscussieerd in **Hoofdstuk 7**. Ik heb laten zien dat voor herbivoor-bloembezoeker interacties variatie in de eigenschappen van de verschillende spelers belangrijk is voor de uitkomst van deze indirecte interacties: variatie in eigenschappen van herbivoren, planten, en bloembezoekers heeft invloed op de dynamiek van de gemeenschap van bloembezoekers en heeft gevolgen voor de fitness van planten. De combinatie van de manier waarop een herbivoor de plant aanvalt en de keuzen voor een bepaalde voedingsplek bepalen deels de richting en sterkte van herbivoor-bestuiver en herbivoor-herbivoor interacties. Herbivoor-geactiveerde veranderingen in bloemkenmerken zijn voor een groot deel specifiek voor de soort herbivoor en daardoor is de identiteit van de belager ook belangrijk en kan de uitkomst van herbivoor-bloembezoeker interacties beïnvloeden. Variatie in planteneigenschappen gedurende de ontwikkeling van de plant vergroot de specificiteit verder, en het netwerk

van indirecte interacties tussen herbivoren en bloembezoekers is dynamische over de ontwikkeling van de plant, met gevolgen voor plantenfitness. Vraatschade kan negatieve maar ook positieve gevolgen hebben voor verschillende componenten van plantenfitness, zowel vrouwelijke (zaadproductie) als mannelijke (stuifmeelproductie) fitness. Het samenspel van direct effecten van vraatschade en indirecte effecten via veranderingen in bestuiver visitatie is waarschijnlijk belangrijk voor plantenfitness en zou moeten worden meegenomen bij overdenking over plantenevolutie.

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Although I can be serious at work (yes, seriously), I regularly need to reset and enjoy other aspects of life besides science (yes, really). I want to thank all my friends outside the university for their support and great times. Bedankt, **Olaf**, **Jos**, **Mathijs**, **Jasper**, **Niels**, **Sjors**. Bedankt voor de heerlijke avondjes voetbal. Bedankt voor bier, samenwonen, zwevende eieren, vliegend glas, ontploffingen, graafmachines om 5 uur 's ochtends, kruidige geurtjes, kamelen, reisjes naar Lanzarote, Maleisië, Boedapest, Stockholm, feestjes, festivals en ramtrapavonden, opblaas Unox-worsten, gefrituurde courgette, de trap van Calzatura, kleine stoeltjes met emotionele waarde, overvolle servieskasten, How To Basic, badkuipen, knäckebröd met dubieuze gaten, zijdelingse foto's en getooste broodjes, Amsterdam Dance Events, Vato Gonzalez en Dirty House, VVSB-FC Utrecht, logeren in Zwolle, marshmallows op de weg, karten, slangen in schoenen, blote billen, ratten en nagels, en nog heel veel meer. Bedankt voor al het lachen, gieren en brullen. Ik hoop dat de afstanden van de toekomst niet tussen ons komen te staan. I want to thank my friends from my hometown for their patience and seeking me out in far-away Wageningen. **Frank**, **Eric**, **Roel**, **Erik**, **Rick**, bedankt voor jullie gezelligheid en altijd interessante gesprekken. Bedankt dat jullie af en toe naar Wageningen komen om lol te trappen, en altijd tijd hebben als ik in de buurt ben. Bedankt voor het onderhouden van onze vriendschap terwijl ik in het verre Wageningen druk bezig ben met bloempjes en bijtjes.

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Jeugd van Tegenwoordig, Kanye West, Childish Gambino, John Frusciante, Therapy?, Triggerfinger, Paramore, Paul Kalkbrenner, Franky Rizardo, Sandeep, Oliver Weiter, Miss Melera, the XX, Liquicity, Maduk, Defected, Sonny Fodera, MK, Ferreck Dawn, Papa Roach, Oasis, Arctic Monkeys, Vato Gonzalez, Snow Patrol, Marbert Rocel, Snow Patrol, Nickelback, Chickenfoot, Goldfish, Kids See Ghosts, Joris Voorn, Prunk, The Magician, Molotov, Gui Boratto, Isaac Tichauer, Mr. Belt and Wezol, Buurman & Buurman – Relativ.

I wish to thank my family for their support, warmth, and being in my life. **Pa** en **Ma**, jullie zijn een voorbeeld voor menige ouders. Bedankt dat jullie me (bijna) altijd mijn gang lieten gaan en mij steunden in al mijn keuzes, ook toen ik naar Wageningen wilde om biologie te gaan studeren. Bedankt voor de fantastische opvoeding die mij gemaakt heeft tot wie ik nu ben, en alle liefde ondanks de afstand. **Joris**, **Stein**, en **Pino**, jullie zijn geweldige broers en zus. Bedankt dat ik altijd bij jullie aan kan kloppen. Bedankt voor alle gezelligheid en buitensporigheid, ik geniet enorm van onze activiteiten en feestjes. Moge deze tot in de eeuwigheid voortduren, net als zekers!, scheve vloeren, en Keizer Kip en de Kaasganzen. **Cathy**, **Kim**, en **Sjors**, bedankt voor de neven en nichtenavondjes en de terror dates, voor de gezelligheid en het helemaal uit de plaat gaan. **Har** en **Marian**, bedankt voor de kerstkaart die elk jaar Wageningen weer weet te vinden, de gezelligheid in Hillegom, en de leuke gesprekken en discussie tussen de Asclepias over planten en insecten in de praktijk. Mogelijk gaan de Asclepias nog een belangrijke rol voor mij spelen. **Gerda** en **Reinier**, bedankt voor het lekkere eten en alle gezelligheid. Ik waardeer het enorm dat jullie mij zo warm opnemen in de familie, al ben ik ver van de ideale schoonzoon.

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About the author

Quint Rusman was born on July 9th, 1991 in Noordwijkerhout, The Netherlands. After finishing high school in Noordwijkerhout, he moved to Wageningen to study Biology at Wageningen University. After finishing his bachelor Biology with a focus on ecology, he started the master programme Biology at Wageningen University, with focus on bio-interactions, entomology and plant ecology. He conducted his first master thesis project under the supervision of Prof. Dr David Kleijn at the Resource Ecology Group of Wageningen University. In this project, the pollination effectiveness of wild and domesticated pollinators in an apple orchard was investigated. His second master thesis project was supervised by Prof. Dr Marcel Dicke of the Laboratory of Entomology at Wageningen University, and Dr Betty Benrey of the Laboratory of Evolutionary Entomology at the University of Neuchatel. In this project, the effect of landscape composition and the presence of alternative hosts on host-parasitoid interactions was investigated. By the end of his MSc programme, Quint enrolled in the Experimental Plant Sciences Graduate Program, a program for excellent master students to write a PhD proposal and apply for funding. Together with Dr Erik H. Poelman, Dr Dani Lucas-Barbosa, and Prof. Dr Marcel Dicke of the Laboratory of Entomology at Wageningen University a proposal entitled: "Dealing with herbivores at the expense of pollination: effects of herbivore-induced changes on flower traits" was written and with success: funding was granted (grant nr. 831.14.004). Starting in 2014 with his PhD project, Quint studied how attack by a range of herbivore species affects plant interactions with mutualistic and antagonistic flower visitors, the underlying herbivore-induced changes in flower traits, and whether these interactions have plant fitness consequences. In a comparative approach using ten different herbivore species, field, greenhouse, and laboratory work were combined. For the future, Quint aims at pursuing a career in academia and continue studying how indirect interactions can drive ecological and evolutionary dynamics of complex communities, with special focus on plants and herbivore – flower-visitor interactions.

Publications

In peer-reviewed journals

Rusman, Q., E. H. Poelman, F. Nowrin, G. Polder, and D. Lucas-Barbosa. **2019**. Floral plasticity: Herbivore-species-specific-induced changes in flower traits with contrasting effects on pollinator behaviour. Plant, Cell & Environment In press. **(chapter 5 in this thesis)**.

Aartsma, Y., Cusumano, A., de Bobadilla, M. F., **Rusman, Q**., Vosteen, I., & Poelman, E. H. **2019**. Understanding insect foraging in complex habitats by comparing trophic levels: insights from specialist host-parasitoid-hyperparasitoid systems. Current Opinion in Insect Science 32: 54-60

Rusman, Q., D. Lucas-Barbosa, and E. H. Poelman. **2018**. Dealing with mutualists and antagonists: Specificity of plant-mediated interactions between herbivores and flower visitors, and consequences for plant fitness. Functional Ecology 32:1022-1035 **(chapter 3 in this thesis)**.

Other publications

Rusman Q., Janssen J., Corporaal A. **2018**. Hoofdstuk 1: Een orchideetje meer of minder. In: JHJ Schaminée & JAM Jansen (red.) Buigen of barsten. Beschouwingen over de veerkracht van de natuur. KNNV Uitgeverij, Zeist.

Rusman, Q. and Haveman, R. **2014** Hoofdstuk 1: Diversiteit en soortvorming in Europese vaatplanten. In: JHJ Schaminée & JAM Jansen (red.) Het oude continent. Beschouwingen over de natuur in Europa. KNNV Uitgeverij, Zeist.

Rusman, Q. and Poelman E.H. **2013**, Effect of succession of three different caterpillar species on *Brassica oleraceae*, plant defense divergence or convergence? Student Research Conference, Amsterdam, The Netherlands.

Submitted

Rusman, Q., D. Lucas-Barbosa, E. H. Poelman, and M. Dicke. **chapter 2**. The ecology of plastic flowers.

Rusman, Q., D. Lucas-Barbosa, K. Hassan, and E. H. Poelman. **chapter 4**. Plant ontogeny determines strength and associated fitness consequences of plant-mediated interactions between herbivores and flower visitors.

Rusman, Q., P. N. Karssemeijer, D. Lucas-Barbosa, and E. H. Poelman. **chapter 6**. Settling on leaves or flowers: Herbivore feeding site determines the outcome of indirect interactions between herbivores and flower visitors.

Appendix A

Chapter 3
Dealing with mutualists and antagonists: Specificity of plant-mediated interactions between herbivores and flower visitors, and consequences for plant fitness

Quint Rusman, Dani Lucas-Barbosa, Erik H. Poelman

Functional Ecology **2018**, 32, 1022-1035

Table A1. Adjusted residuals for the number of visitors of each pollinator group (honeybees, bumblebees, syrphid flies and solitary bees) for uninfested *Brassica nigra* plants or plants infested with chewing, sap-feeding or root herbivores. Numbers displayed in bold exceed the ± 2 criteria.

	Uninfested (control)	Chewing herbivores	Sap-feeding herbivores	Root herbivores
Honeybees	-0.848	-0.302	1.595	-0.695
Bumblebees	**-2.178**	-0.080	-1.338	**2.808**
Syrphid flies	**2.906**	-0.501	-0.617	-0.752
Solitary bees	-1.164	**2.044**	-0.820	-0.599

Table A2. Output of (generalized) linear (mixed) models showing the effects of different fixed (herbivore treatment, herbivore functional group, and time point) factors on attraction and visitation of different pollinator groups (honeybees, bumblebees, and syrphid flies), and abundance of pollen beetle adults (*Meligethes aeneus*). Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

		Herbivore treatment (T)			Herbivore Functional Group (HFG)			Time point(TP)			T*TP			HFG*TP		
		df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
All pollinators	Number of visitors	7	3.88	0.793	3	6.15	0.104	1	4.85	**0.028**	7	10.87	0.145	3	0.61	0.894
	Flowers visited in total	7	5.11	0.647	3	1.01	0.800	1	2.14	0.144	7	7.61	0.368	3	4.08	0.253
Honeybees	Number of visitors	7	2.59	0.920	3	3.58	0.310	1	6.29	**0.012**	7	2.92	0.892	3	0.387	0.943
	Flowers visited in total	7	3.99	0.781	3	0.10	0.991	1	7.42	**0.006**	7	6.37	0.497	3	4.14	0.246
	Flowers visited per visit	7	3.72	0.812	3	0.76	0.860	1	0.040	0.841	7	4.25	0.751	3	2.18	0.536
	Time spend per plant	7	4.64	0.704	3	0.932	0.818	1	0.038	0.845	10	27.27	**0.002**	3	6.12	0.106
	Time spend per flower	7	0.69	0.998	3	3.27	0.351	1	1.46	0.227	7	12.50	*0.085*	3	0.51	0.918
Bumblebees	Number of visitors	7	2.58	0.921	3	4.35	0.226	1	1.56	0.212	7	10.98	0.139	3	5.17	0.160
Syrphid flies	Number of visitors	7	7.80	0.351	3	10.78	**0.013**	1	16.41	**<0.001**	7	7.33	0.396	3	0.92	0.820
	Flowers visited in total	7	4.95	0.666	3	5.16	0.161	-	-	-	-	-	-	-	-	-
	Flowers visited per visit	7	6.59	0.473	3	2.76	0.431	-	-	-	-	-	-	-	-	-
	Time spend per plant	7	14.42	**0.044**	3	6.34	*0.096*	-	-	-	-	-	-	-	-	-
	Time spend per flower	7	11.94	0.103	3	5.15	0.161	-	-	-	-	-	-	-	-	-
Pollen beetle adults	Number of visitors	7	4.39	0.734	3	2.12	0.547	1	0.092	0.761	7	8.60	0.283	3	5.15	0.161

Table A3. Output of (generalized) linear (mixed) models showing the effects of different random factors on attraction and visitation of different pollinator groups (honeybees, bumblebees, and syrphid flies), and abundance of pollen beetle adults (*Meligethes aeneus*). All random factors were initially included in the model, and factors which explained less than 10^{-2} variation or with a *P* - value above 0.05 were excluded from the model. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

		Time			Block (1 vs 2)			Day (D)			Plot			D*T			D*HFG		
		df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
All pollinators	Number of visitors	1	44.49	**<0.001**	1	3.84	**0.050**	1	89.52	**<0.001**	1	56.31	**<0.001**	-	-	-	-	-	-
	Flowers visited in total	-	-	-	-	-		1	32.03	**<0.001**	1	77.47	**<0.001**	1	223.56	**<0.001**	**-**	-	**-**
Honeybees	Number of visitors	1	33.40	**<0.001**	1	3.18	*0.075*	1	39.83	**<0.001**	1	19.93	**<0.001**	1	3.88	**0.049**	1	0.69	0.406
	Flowers visited in total	-	-	-	-	-	-	1	0.70	0.403	1	233.37	**<0.001**	1	117.73	**<0.001**	1	10.33	**0.001**
	Flowers visited per visit	-	-	-	-	-	-	1	9.32	**0.002**	1	14.91	**<0.001**	1	24.34	**<0.001**	-	-	-
	Time spend per plant	-	-	-	-	-	-	1	3.70	*0.054*	1	4.74	**0.029**	1	0.26	0.611	-	-	-
	Time spend per flower	1	3.52	*0.060*	1	<0.01	0.954	1	31.59	**<0.001**	1	0	1	-	-	-	-	-	-
Bumblebees	Number of visitors	1	1.54	0.215	-	-	-	1	2.93	*0.087*	-	-	-	-	-	-	-	-	-
Syrphid flies	Number of visitors	1	21.19	**<0.001**	1	39.24	**<0.001**	1	3.01	*0.083*	-	-	-	-	-	-	-	-	-
	Flowers visited in total	-	-	-	-	-	-	1	11.26	**<0.001**	1	8.61	**0.003**	1	13.19	**<0.001**	**-**	-	**-**
	Flowers visited per visit	-	-	-	-	-	-	1	9.25	**0.002**	1	16.75	**<0.001**	1	5.17	**0.023**	**-**	-	**-**
	Time spend per plant	1	0	1	-	-	-	1	5.55	**0.019**	1	0	1		-	-	-	-	-
	Time spend per flower	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Pollen beetle adults	Number of visitors	-	-	-	1	1.59	0.208	1	140.67	**<0.001**	1	47.55	**<0.001**	1	22.66	**<0.001**	-	-	-

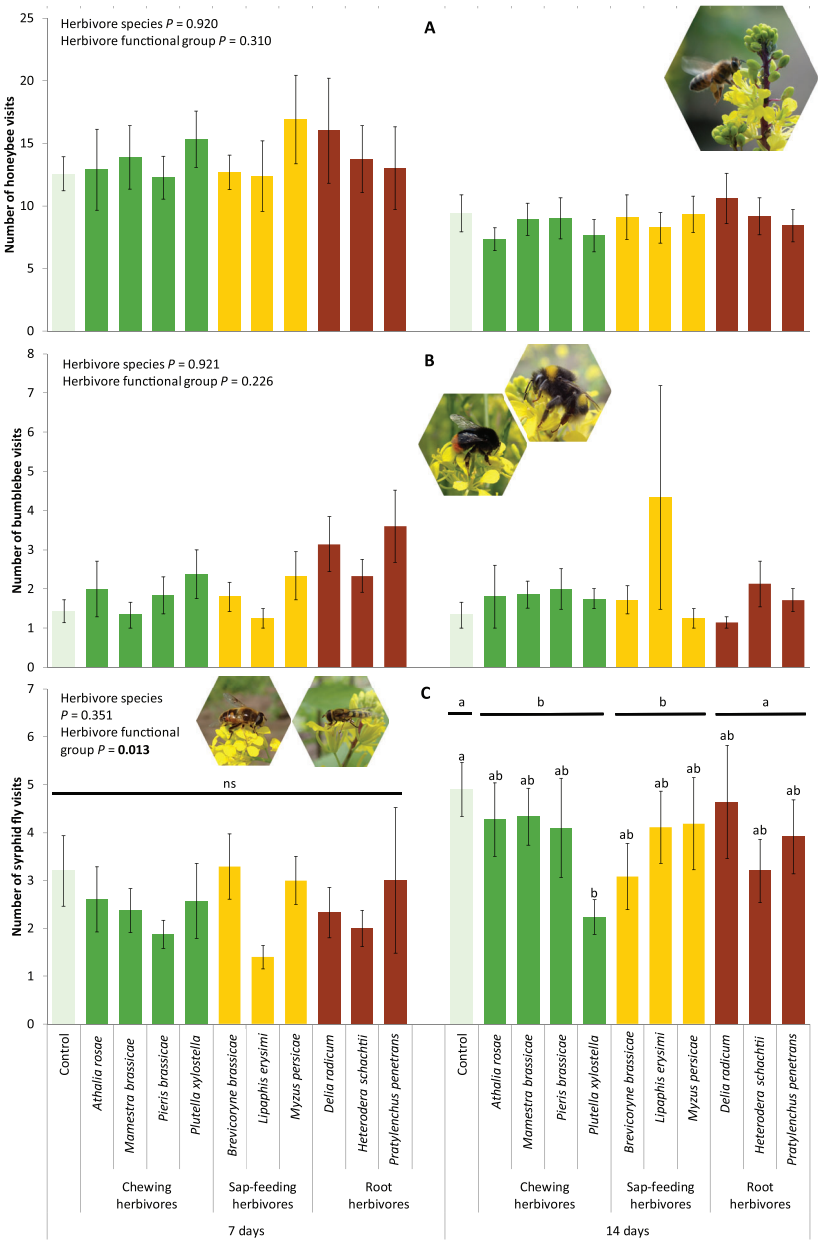


Fig. A1 Number of pollinator visits observed (mean ± SE) on flowers of uninfested plots (control) of *Brassica nigra* and on flowers of plots of plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs): chewing, sap-feeding or root herbivory. We observed visitation by A) honeybees, B) bumblebees, C) syrphid flies. Observations lasted for 10 minutes and were made at two time points: 7 and 14 days after infestation. Number of replicates per herbivore treatment varied between 8 and 13 for honeybees (A), 3 and 8 for bumblebees (B), 5 and 13 for syrphid flies (C). Letters above bars indicate significant differences at $\alpha \leq 0.05$ based on Tukey's *post hoc* tests.

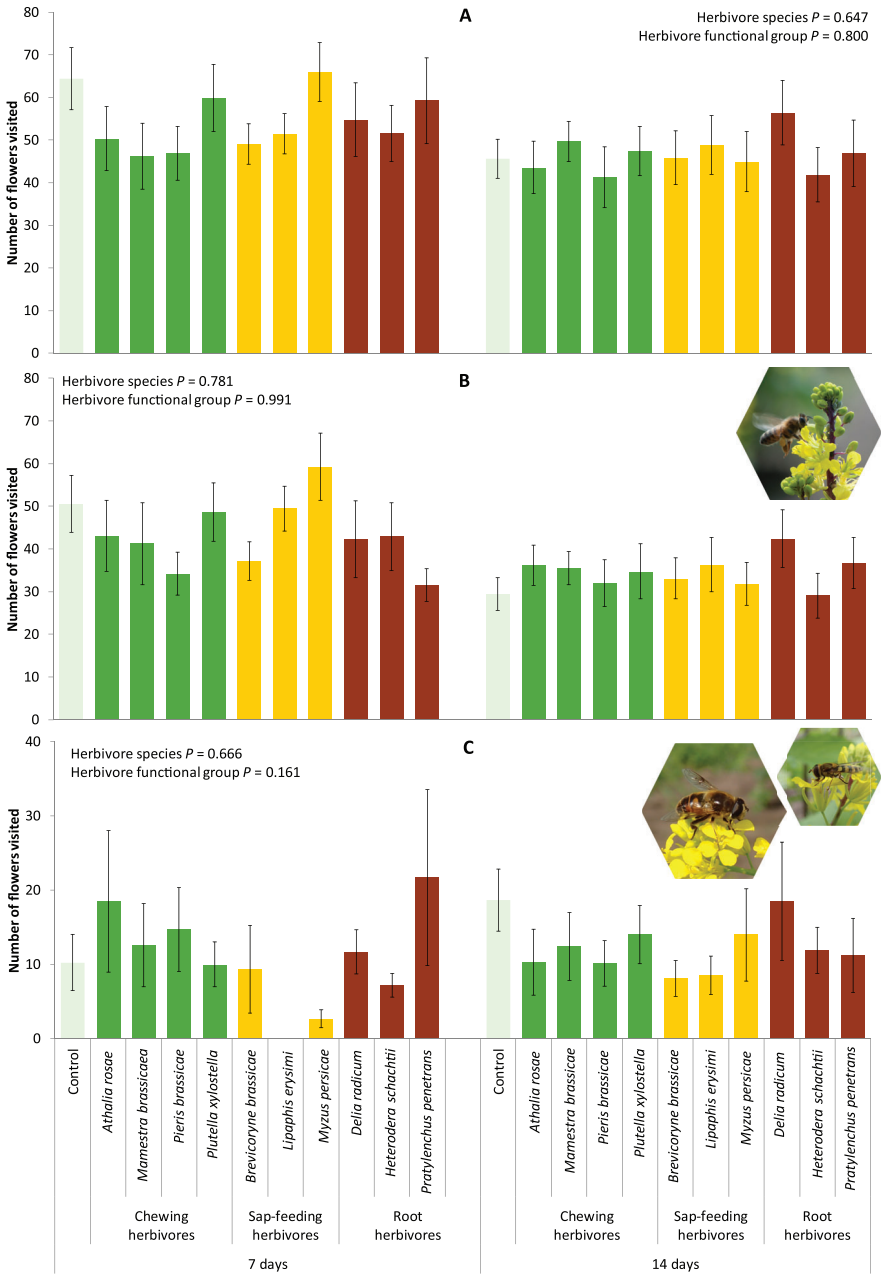


Fig. A2 Number of flowers visited (mean ± SE) of uninfested plots (control) of *Brassica nigra* and of plots of plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs): chewing, sap-feeding or root herbivory. We observed flower visitation by A) all pollinators, B) honeybees, C) syrphid flies. Observations lasted for 10 minutes and were made at two time points: 7 and 14 days after infestation. Number of replicates per herbivore treatment varied between 7 and 13 for all pollinators (A), 7 and 13 for honeybees (B), 0 and 11 for syrphid flies (C).

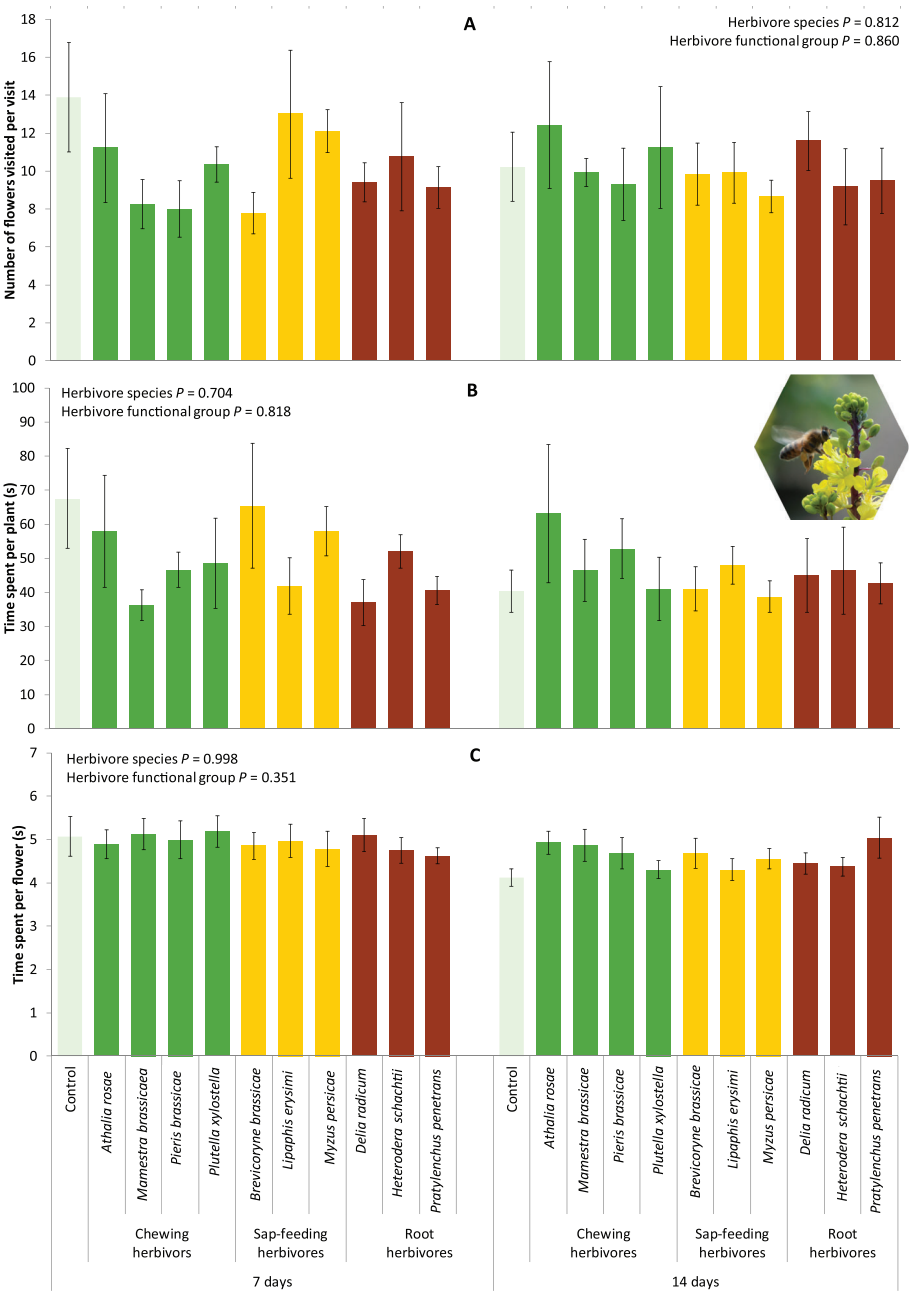


Fig. A3 A) Number of flowers visited per visit (mean ± SE), B) Time spent per plant (s), C) Time spent per flower (s) by honeybees visiting either uninfested plots (control) of *Brassica nigra* or plots of plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs): chewing, sap-feeding or root herbivory. Observations lasted for 10 minutes and were made at two time points: 7 and 14 days after infestation. Number of replicates per herbivore treatment varied between 7 and 13.

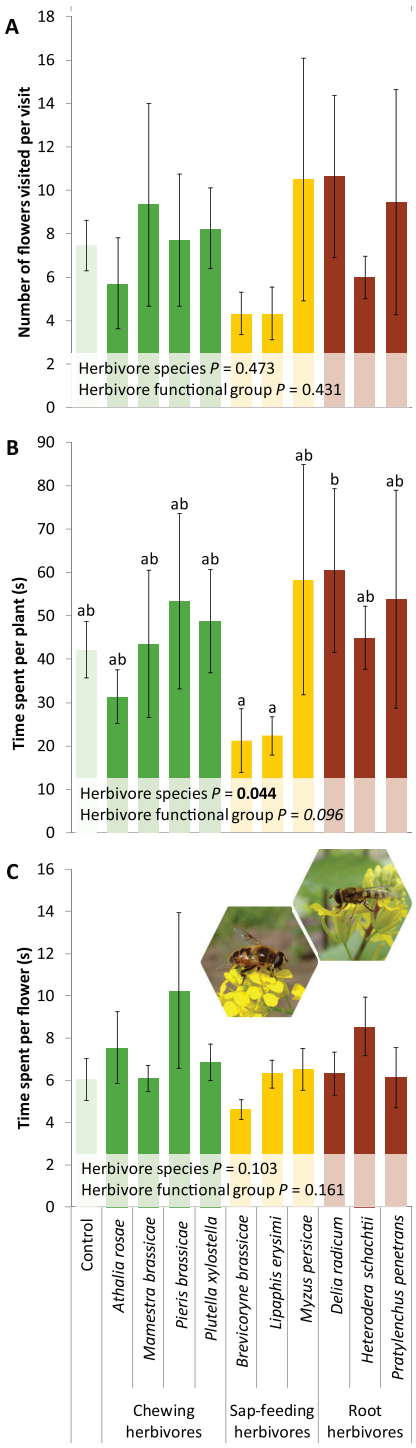


Fig. A4 A) Number of flowers visited per visit (mean ± SE), B) Time spent per plant (s), C) Time spent per flower (s) by syrphid flies visiting either uninfested plots (control) of *Brassica nigra* or of plots of plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs): chewing, sap-feeding or root herbivory. Observations lasted for 10 minutes and were made 14 days after infestation. Number of replicates per herbivore treatment varied between 7 and 11. Letters above bars indicate significant differences at $\alpha \leq 0.05$ based on Tukey's *post hoc* tests.

Table A4. Output of (generalized) linear mixed models showing the effects of fixed (herbivore treatment or herbivore functional group (HFGs)) and random factors on pollen beetle (*Meligethes aeneus*) larvae abundance and performance. Random factors such as day and plot were initially included in the model, and factors which explained less than 10^{-2} variation or with a *P* - value above 0.05 were excluded from the model. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

	Fixed factor						Random factors											
	Herbivore treatment (T)			Herbivore functional group (HFG)			Day			Plot			Day*T			Day*HFG		
	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
Total number	7	5.30	0.624	3	1.87	0.600	1	3.44	*0.064*	-	-	-	1	456.94	**<0.001**	-	-	-
Total weight	8	12.52	0.129	3	11.16	**0.011**	1	19.86	**<0.001**	1	56.27	**<0.001**	-	-	-	-	-	-
Number 1st instar	7	7.53	0.376	3	3.99	0.262	1	13.41	**<0.001**	-	-	-	1	51.64	**<0.001**	-	-	-
Weight 1st instar	7	27.07	**<0.001**	3	2.68	0.444	-	-	-	-	-	-	-	-	-	-	-	-
Number 2nd instar	7	4.29	0.746	3	2.52	0.473	1	1.02	0.314	-	-	-	1	332.04	**<0.001**	-	-	-
Weight 2nd instar	8	16.09	**0.041**	3	10.32	**0.016**	1	11.03	**<0.001**	1	41.09	**<0.001**	-	-	-	-	-	-

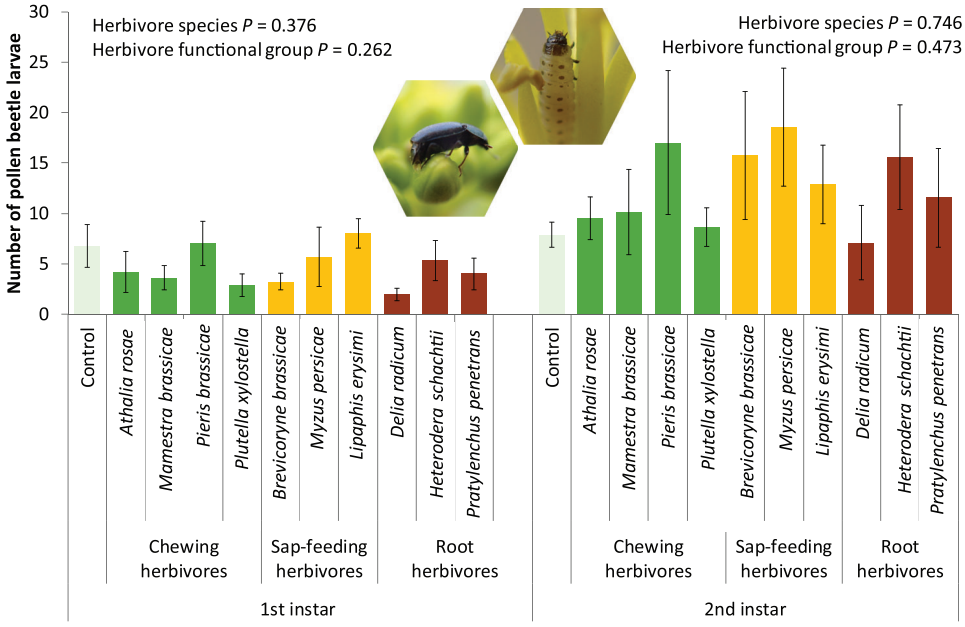


Fig. A5 Number of 1st and 2nd instar larvae of the pollen beetle *Meligethes aeneus* (mean ± SE) found in buds of uninfested (control) *Brassica nigra* plants and of plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs); chewing, sucking or root herbivory, which were exposed to pollen beetle adults. Number of replicates per herbivore treatment varied between 7 and 10.

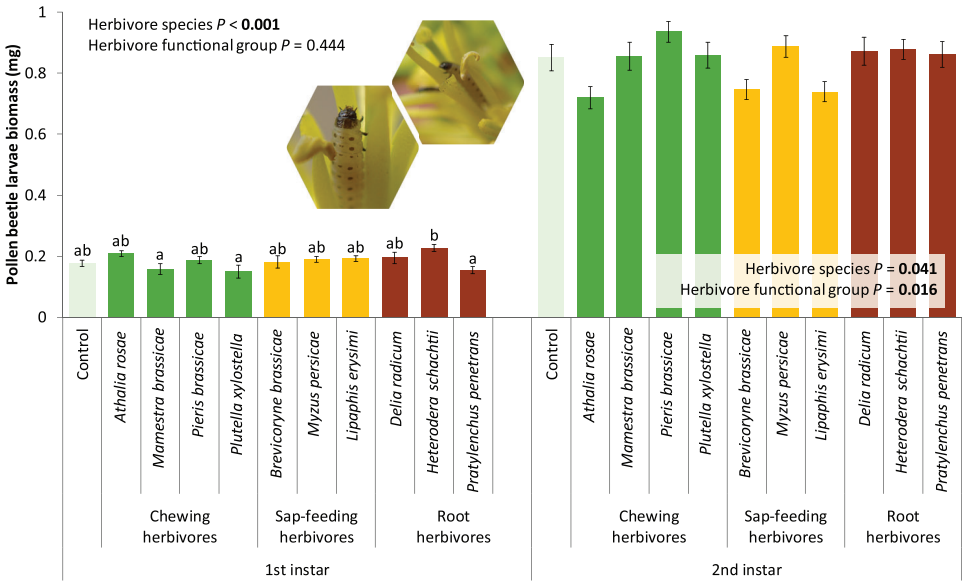


Fig. A6 Biomass of 1st and 2nd instar larvae of the pollen beetle *Meligethes aeneus* (mean ± SE) found in buds of uninfested (control) *Brassica nigra* plants and of plants infested by different herbivores that belong to one of three different herbivore functional groups (HFGs); chewing, sucking or root herbivory, which were exposed to pollen beetle adults. Number of replicates per herbivore treatment varied between 18 and 56 for 1st instar larvae, and between 56 and 144 for 2nd instar larvae. Letters above bars indicate significant differences at $P \leq 0.05$ based on Tukey's *post hoc* tests.

Table A5. Output of generalized linear mixed models showing the effects of fixed (herbivore treatment or herbivore functional group (HFGs)) and random factors on plant seed set, for a plot composed of 5 plants. The random factor block was included in the model, and factors which explained less than 10^{-3} variation or with a *P* - value above 0.05 were excluded from the model. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

	Fixed factors						Random factor					
	Herbivore species (T)			Herbivore functional groups (HFG)			Block			Block *T		
	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
Number of seeds per plot	7	7.72	0.358	3	5.84	0.119	1	588937	**<0.001**	-	-	-
Number of seeds per central plant	7	11.17	0.131	3	13.07	**0.004**	1	125239	**<0.001**	-	-	-
Number of seeds per side plant	`7	5.29	0.624	3	2.37	0.499	1	43.39	**<0.001**	1	19672	**<0.001**

Appendix B

Chapter 4
Plant ontogeny determines strength and associated plant fitness consequences of plant-mediated interactions between herbivores and flower visitors

Quint Rusman, Dani Lucas-Barbosa, Kamrul Hassan, Erik H. Poelman

Submitted

Table B1. Output of generalized linear (mixed) models showing the effects of different fixed (herbivore species and plant ontogenetic stage) factors on plant phenological traits. All random factors were initially included in the model, and factors which explained less than 3 percent variation or with a *P* - value above 0.05 were excluded from the model. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

	Herbivore species (T)			Plant ontogenetic stage (O)			T*D			Plot			Plant position		
	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
Days to reach bud stage	6	13.70	**0.033**	-	-	-	-	-	-	-	-	-	-	-	-
Length of bud stage	6	66.55	**<0.001**	1	10.19	**0.001**	5	45.15	**<0.001**	-	-	-	-	-	-
Vegetative stage	6	112.01	**<0.001**	-	-	-	-	-	-	-	-	-	-	-	-
Budding stage	6	4.34	0.631	-	-	-	-	-	-	-	-	-	-	-	-
Days to reach flowering stage	6	24.06	**<0.001**	1	0.93	0.33	5	34.28	**<0.001**	-	-	-	-	-	-
Vegetative stage	6	57.33	**<0.001**	-	-	-	-	-	-	-	-	-	-	-	-
Bud stage	6	1.31	0.971	-	-	-	-	-	-	-	-	-	-	-	-
Length of the flowering stage	6	24.91	**<0.001**	2	5.47	*0.065*	10	39.67	**<0.001**	-	-	-	-	-	-
Vegetative stage	6	23.13	**<0.001**	-	-	-	-	-	-	-	-	-	-	-	-
Bud stage	6	18.30	**0.006**	-	-	-	-	-	-	-	-	-	-	-	-
Flowering stage	6	25.73	**<0.001**	-	-	-	-	-	-	-	-	-	-	-	-
Lifetime	6	20.37	**0.002**	2	5.27	*0.072*	10	36.50	**<0.001**	-	-	-	-	-	-
Vegetative stage	6	45.38	**<0.001**	-	-	-	-	-	-	-	-	-	-	-	-
Bud stage	6	7.64	0.266	-	-	-	-	-	-	-	-	-	-	-	-
Flowering stage	6	11.55	*0.073*	-	-	-	-	-	-	-	-	-	-	-	-

Table B2. Comparisons between *Brassica nigra* plants exposed to herbivores in different plants ontogenetic stages (vegetative, bud, flowering) for various plant traits and insect measurements on different time points. Comparison were performed with Tukey's post hoc tests after initial modelling with (generalized) linear (mixed) models. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

	Vegetative-bud	Vegetative-flowering	Bud-flowering	Vegetative-bud	Vegetative-flowering	Bud-flowering
	Length of budding stage			Days to reach the flowering stage		
Pieris brassicae	**<0.001**	-	-	**<0.001**	-	-
Athalia rosae	0.881	-	-	0.653	-	-
Brevicoryne brassicae	0.258	-	-	*0.085*	-	-
Lipaphis erysimi	0.912	-	-	0.609	-	-
Delia radicum	0.576	-	-	**0.048**	-	-
Heterodera schachtii	0.553	-	-	**0.010**	-	-
	Flowering time			Lifetime		
Pieris brassicae	0.917	0.118	**0.048**	**0.005**	0.182	0.353
Athalia rosae	0.822	0.908	0.981	0.686	0.877	0.935
Brevicoryne brassicae	**0.032**	0.142	0.796	**0.005**	**0.016**	0.921
Lipaphis erysimi	**0.002**	0.452	**<0.001**	0.525	*0.056*	**0.003**
Delia radicum	0.203	0.739	**0.033**	*0.056*	0.543	0.417
Heterodera schachtii	0.207	0.443	0.880	0.269	0.492	0.910

	Vegetative-bud	Vegetative-flowering	Bud-flowering	Vegetative-bud	Vegetative-flowering	Bud-flowering
	Number of inflorescences 1 week after plants started to produce buds			Number of inflorescences 2 weeks after the start of flowering		
Pieris brassicae	**<0.001**	-	-	**<0.001**	**0.001**	0.981
Athalia rosae	*0.053*	-	-	0.274	0.989	0.317
Brevicoryne brassicae	0.363	-	-	**0.021**	**0.008**	0.901
Lipaphis erysimi	0.105	-	-	*0.092*	0.961	*0.066*
Delia radicum	0.318	-	-	0.932	0.334	0.205
Heterodera schachtii	*0.085*	-	-	0.393	0.278	0.961
	Pollinator community					
Pieris brassicae	0.762	0.762	0.934			
Athalia rosae	0.921	0.921	0.921			
Brevicoryne brassicae	0.276	0.276	0.276			
Lipaphis erysimi	0.284	0.392	0.392			
Delia radicum	**0.004**	0.598	**0.006**			
Heterodera schachtii	*0.095*	*0.095*	0.877			
	Number of pollinators 1 week after the start of flowering			Number of pollinators 2 weeks after the start of flowering		
Pieris brassicae	*0.078*	0.400	0.659	**0.004**	**0.048**	0.601
Athalia rosae	0.516	0.283	**0.025**	**0.002**	0.847	**0.010**
Brevicoryne brassicae	0.213	0.336	0.957	*0.086*	*0.094*	0.987
Lipaphis erysimi	**0.007**	0.992	**0.007**	0.446	0.987	0.575
Delia radicum	0.834	**0.011**	**0.002**	0.454	0.949	0.701
Heterodera schachtii	**0.002**	0.786	**<0.001**	**0.040**	0.857	0.118
	Number of honeybees 1 week after the start of flowering			Number of honeybees 2 weeks after the start of flowering		
Pieris brassicae	**0.007**	*0.097*	0.603	**0.004**	**0.041**	0.638
Athalia rosae	0.403	0.443	**0.034**	**0.003**	0.866	**0.012**
Brevicoryne brassicae	0.164	0.409	0.839	0.167	0.154	0.998
Lipaphis erysimi	0.145	0.988	0.121	0.599	1.000	0.620
Delia radicum	0.954	**0.021**	**0.012**	0.345	0.984	0.498
Heterodera schachtii	**0.021**	0.329	**<0.001**	**0.030**	0.809	0.108
	Number of syrphid flies 1 week after the start of flowering			Number of syrphid flies 2 weeks after the start of flowering		
Pieris brassicae	0.852	0.688	0.951	0.421	0.974	0.110
Athalia rosae	0.992	0.792	0.719	0.205	0.985	0.258
Brevicoryne brassicae	0.461	0.329	0.968	*0.054*	0.118	0.887
Lipaphis erysimi	0.123	0.828	**0.016**	**0.011**	0.258	0.319
Delia radicum	0.596	0.696	0.177	0.306	0.431	0.986
Heterodera schachtii	0.134	0.699	0.411	*0.071*	0.989	*0.054*
	Number of flowers visited by pollinators 1 week after the start of flowering			Number of flowers visited by pollinators 2 weeks after the start of flowering		
Pieris brassicae	0.965	0.331	0.215	0.690	*0.080*	0.228
Athalia rosae	0.242	0.272	**0.005**	0.989	0.584	0.577
Brevicoryne brassicae	0.348	**0.001**	*0.057*	0.923	0.298	0.595
Lipaphis erysimi	0.129	0.974	0.219	0.211	**0.046**	0.789
Delia radicum	0.897	**0.010**	**0.003**	0.318	**0.024**	0.361
Heterodera schachtii	0.322	0.547	0.919	0.292	0.276	0.935
	Number of flowers visited by honeybees 1 week after the start of flowering			Number of flowers visited by honeybees 2 weeks after the start of flowering		
Pieris brassicae	0.141	0.314	**0.002**	0.770	0.159	0.334
Athalia rosae	0.222	0.423	**0.010**	0.988	0.836	0.760
Brevicoryne brassicae	0.494	**0.031**	0.318	0.977	0.673	0.600
Lipaphis erysimi	0.551	0.578	0.999	0.374	0.215	0.945
Delia radicum	0.998	**0.016**	**0.026**	0.194	*0.064*	0.757

Table B3. Output of generalized linear (mixed) models showing the effects of different fixed (herbivore species and plant ontogenetic stage) factors on flower abundance, attraction and visitation by pollinators, and abundance of pollen beetle adults for different time-points. All random factors were initially included in the model, and factors which explained less than 3 percent variation or with a *P*- value above 0.05 were excluded from the model. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

		Herbivore treatment (T)			Plant ontogenetic stage (O)			T*O			Time			Day			Day*T / Plot (Inflor. + PB)			Plant position			Observer		
One week after plants started to produce buds		df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
Inflorescences	Number	5	25.11	**<0.001**	1	8.36	**0.004**	5	28.54	**<0.001**	-	-	-	-	-	-	1	95.62	**<0.001**	-	-	-			
Pollen beetle adults	Number of visitors	5	11.61	**0.041**	1	4.75	**0.029**	5	25.04	**<0.001**	-	-	-	1	75.30	**<0.001**	1	38.68	**<0.001**	-	-	-			
One week after the start of flowering																									
Inflorescences	Number	5	1.66	0.894	2	0.05	0.975	10	2.32	0.993	-	-	-	-	-	-	1	35.19	**<0.001**	-	-	-	-	-	-
All pollinators	Number of visitors	5	6.21	0.286	2	1.04	0.594	10	41.75	**<0.001**	-	-	-	1	11.64	**<0.001**	1	62.69	**<0.001**	-	-	-	-	-	-
	Flowers visited in total	5	8.33	0.139	1	2.30	0.317	10	13.00	0.224	-	-	-	1	5.41	**0.020**	1	0	1	-	-	-	1	19.40	**<0.001**
Honeybees	Number of visitors	5	7.31	0.199	2	1.66	0.437	10	32.90	**<0.001**	-	-	-	1	8.35	**0.004**	1	55.44	**<0.001**	-	-	-	-	-	-
	Flowers visited in total	5	9.74	*0.083*	2	2.52	0.283	10	7.05	0.721	-	-	-	1	1.94	0.167	1	0	1	-	-	-	1	15.08	**<0.001**
	Flowers visited per visit	5	3.42	0.635	2	4.87	*0.087*	10	6.21	0.797	-	-	-	1	0.19	0.664	-	-	-	-	-	-	-	-	-
	Time spend per plant	5	0.441	0.994	2	2.06	0.356	10	8.59	0.572	1	0	0.989	-	-	-	1	9.03	**0.003**	-	-	-	1	0.38	0.536
	Time spend per flower	5	0.463	0.993	2	4.95	*0.084*	10	17.90	*0.058*	-	-	-	1	3.77	*0.052*	1	10.42	**0.001**	-	-	-	1	17.37	**<0.001**
Bumblebees	Number of visitors	5	5.20	0.392	2	2.22	0.329	9	2.59	0.978	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Syrphid flies	Number of visitors	5	4.36	0.499	2	6.50	**0.039**	10	9.81	0.457	-	-	-	1	49.07	**<0.001**	1	3.25	*0.071*	-	-	-	-	-	-
	Flowers visited in total	5	6.46	0.264	2	2.84	0.242	10	17.03	*0.074*	-	-	-	1	0.96	0.328	1	2.56	0.110	-	-	-	1	0	1
	Flowers visited per visit	5	15.40	**0.009**	2	6.91	**0.032**	10	14.82	0.139	-	-	-	1	0	1	-	-	-	-	-	-	1	0	1
	Time spend per plant	5	10.44	*0.064*	2	0.80	0.670	10	10.64	0.386	1	0	1	1	6.90	**0.009**	1	0	1	-	-	-	1	0	1
	Time spend per flower	5	9.21	0.101	2	12.64	**0.002**	10	4.90	0.898	-	-	-	1	0.02	0.899	-	-	-	-	-	-	-	-	-
Pollen beetle adults	Number of visitors	5	5.19	0.394	2	7.36	**0.025**	10	20.37	**0.026**	-	-	-	1	13.87	**<0.001**	1	9.61	**0.002**	1	17.38	**<0.001**	-	-	-

		Herbivore treatment (T)			Plant ontogenetic stage (O)			T*O			Time			Day			Day*T / Plot (Inflor. + PB)			Plant position			Observer		
Two weeks after the start of flowering		df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
Inflorescences	Number	5	4.07	0.540	2	3.07	0.215	10	21.63	**0.017**	-	-	-	-	-	-	1	9.17	**0.002**	**-**	**-**	**-**	**-**	**-**	**-**
All pollinators	Number of visitors	5	12.09	**0.034**	2	5.56	*0.062*	10	18.72	**0.044**	-	-	-	1	25.59	**<0.001**	1	0	1	-	-	-	-	-	-
	Flowers visited in total	5	7.06	0.216	2	7.16	**0.028**	10	1.81	0.998	1	0	1	1	0.829	0.363	1	0	1	-	-	-	1	5.50	**0.019**
Honeybees	Number of visitors	5	11.52	**0.042**	2	6.47	**0.039**	10	17.33	*0.067*	-	-	-	1	19.61	**<0.001**	1	0	1	-	-	-	-	-	-
	Flowers visited in total	5	8.44	0.134	2	9.15	**0.010**	10	4.73	0.908	1	0	0.999	-	-	-	1	0	1	-	-	-	1	3.50	*0.061*
	Flowers visited per visit	5	3.08	0.687	2	8.40	**0.015**	10	15.35	0.120	-	-	-	1	1.42	0.233	1	82.65	**<0.001**	-	-	-	-	-	-
	Time spend per plant	5	2.61	0.760	2	0.04	0.981	10	6.57	0.765	1	0	**1**	-	-	-	-	-	-	-	-	-	-	-	-
	Time spend per flower	5	6.99	0.221	2	11.05	**0.004**	10	10.45	0.402	-	-	-	-	-	-	-	-	-	-	-	-	1	0.31	0.579
Bumblebees	Number of visitors*	-	-	-	2	4.53	0.104	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Syrphid flies	Number of visitors	5	17.81	**0.003**	2	0.800	0.670	10	6.66	0.758	-	-	-	1	85.85	**<0.001**	-	-	-	-	-	-	-	-	-
	Flowers visited in total*	-	-	-	3	1.55	0.671	-	-	-	-	-	-	1	0	1	-	-	-	-	-	-	-	-	-
	Flowers visited per visit*	-	-	-	3	0.37	0.946	-	-	-	1	0.03	0.860	1	0.31	0.577	-	-	-	-	-	-	1	0	1
	Time spend per plant*	-	-	**-**	3	6.10	0.107	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	1	0	1
	Time spend per flower*	-	-	-	3	4.38	0.223	-	-	-	1	0	1	-	-	-	-	-	-	-	**-**	-	1	0	1
Pollen beetle adults	Number of visitors	5	7.30	0.200	2	0.10	0.953	10	17.92	*0.056*	-	-	-	1	59.23	**<0.001**	1	16.74	**<0.001**	1	15.46	**<0.001**	-	-	-

Table B4. Output of generalized linear (mixed) models showing the effects of different fixed (herbivore species and time-point) factors on flower abundance, attraction and visitation by pollinators, and abundance of pollen beetle adults, for herbivore exposure in different plant ontogenetic stages. All random factors were initially included in the model, and factors which explained less than 0.03 variation or with a *P*- value above 0.05 were excluded from the model. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

		Herbivore treatment (T)			Time point (TP)			T*TP			Time			Day			Day*T / Plot (Inflor. + PB)			Plant position			Observer		
Vegetative stage		df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
Inflorescences	Number	6	24.22	**<0.001**	2	734.83	**<0.001**	12	139.52	**<0.001**	-	-	-	-	-	-	1	22.72	**<0.001**	-	-	-	-	-	-
All pollinators	Number of visitors	6	9.08	0.169	1	5.14	**0.024**	6	30.65	**<0.001**	-	-	-	1	7.75	**0.005**	1	88.34	**<0.001**	-	-	-	-	-	-
	Flowers visited in total	6	2.60	0.857	1	1.58	0.209	6	4.58	0.598	-	-	-	1	1.12	0.290	1	0	1	-	-	-	1	9.79	**0.002**
Honeybees	Number of visitors	6	8.23	0.222	1	3.58	*0.059*	6	24.11	**<0.001**	-	-	-	1	4.40	**0.036**	1	78.02	**<0.001**	-	-	-	-	-	-
	Flowers visited in total	6	4.28	0.639	1	3.47	*0.062*	6	5.78	0.449	-	-	-	1	0.24	0.623	1	0	1	-	-	-	1	11.83	**<0.001**
	Flowers visited per visit	6	3.70	0.717	1	8.28	**0.004**	6	23.16	**<0.001**	-	-	-	1	2.23	0.135	1	256.79	**<0.001**	-	-	-	-	-	-
	Time spend per plant	6	4.17	0.654	1	0.07	0.786	6	2.29	0.891	-	-	-	1	0.72	0.396	1	0	1	-	-	-	-	-	-
	Time spend per flower	6	3.84	0.698	1	0.115	0.735	6	12.65	**0.049**	-	-	-	1	0	1	-	-	-	-	-	-	1	4.36	**0.037**
Bumblebees	Number of visitors	6	0.714	0.994	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Syrphid flies	Number of visitors	6	12.41	*0.054*	1	6.17	**0.013**	6	3.63	0.726	-	-	-	1	32.13	**<0.001**	1	1.96	0.161	-	-	-	-	-	-
	Flowers visited in total*	6	8.32	0.215	-	-	-	-	-	-	-	-	-	1	0.18	0.672	1	345.23	**<0.001**	-	-	-	1	7.91	**0.005**
	Flowers visited per visit*	6	48.80	**<0.001**	-	-	-	-	-	-	-	-	-	1	145.51	**<0.001**	1	2.60	0.107	-	-	-	1	17.59	**<0.001**
	Time spend per plant*	6	10.44	*0.064*	-	-	-	-	-	-	-	-	-	1	12.60	**<0.001**	1	0	1	-	-	-	1	1.78	0.182
	Time spend per flower*	6	13.46	**0.036**	-	-	-	-	-	-	1	1.30	0.255	-	-	-	-	-	-	-	-	-	-	-	-
Pollen beetle adults	Number of visitors	6	5.80	0.446	2	36.79	**<0.001**	12	63.67	**<0.001**	-	-	-	1	208.89	**<0.001**	1	23.61	**<0.001**	1	16.56	**<0.001**	-	-	-
Bud stage																									
Inflorescences	Number	6	4.91	0.555	2	591.74	**<0.001**	12	29.64	**0.003**	-	-	-	-	-	-	1	15.08	**<0.001**	-	-	-	-	-	-
All pollinators	Number of visitors	6	4.38	0.626	1	2.00	0.157	6	16.91	**0.010**	1	13.75	**<0.001**	1	14.96	**<0.001**	1	69.04	**<0.001**	-	-	-	1	27.62	**<0.001**
	Flowers visited in total	6	10.80	*0.095*	1	2.70	0.101	6	10.19	0.117	-	-	-	1	0.02	0.883	1	0	0.998	-	-	-	1	12.50	**<0.001**
Honeybees	Number of visitors	6	5.26	0.510	1	11.20	**<0.001**	6	3.71	0.715	-	-	-	1	17.66	**<0.001**	1	0	1	-	-	-	1	11.74	**<0.001**
	Flowers visited in total	6	5.63	0.466	1	5.35	**0.021**	6	11.43	*0.076*	-	-	-	1	0.01	0.936	1	0	0.995	-	-	-	1	15.93	**<0.001**
	Flowers visited per visit	6	0.89	0.989	1	2.39	0.122	6	5.82	0.444	-	-	-	1	5.66	**0.017**	1	56.46	**<0.001**	-	-	-	-	-	-

		Herbivore treatment (T)			Time point (TP)			T*TP			Time			Day			Day*T / Plot (Inflor. + PB)			Plant position			Observer		
Bud stage		df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
	Time spend per plant	6	4.06	0.669	1	0.27	0.600	6	6.68	0.352	1	2.59	0.108	1	13.66	**<0.001**	1	0.367	0.545	-	-	-	-	-	-
	Time spend per flower	6	19.89	**0.003**	1	2.58	0.108	6	6.75	0.345	-	-	-	1	0.73	0.394	-	-	-	-	-	-	1	3.45	*0.063*
Bumblebees	Number of visitors	6	6.32	0.388	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Syrphid flies	Number of visitors	6	4.40	0.623	1	6.84	**0.009**	6	14.10	**0.029**	-	-	-	1	12.55	**<0.001**	1	4.86	**0.027**	-	-	-	-	-	-
	Flowers visited in total*	6	6.15	0.407	-	-	-	-	-	-	-	-	-	1	4.31	**0.038**	1	106.91	**<0.001**	-	-	-	1	11.98	**<0.001**
	Flowers visited per visit*	6	79.34	**<0.001**	-	-	-	-	-	-	-	-	-	1	61.42	**<0.001**	-	-	-	-	-	-	1	31.62	**<0.001**
	Time spend per plant*	6	9.43	0.151	-	-	-	-	-	-	-	-	-	1	4.29	**0.038**	-	-	-	-	-	-	1	0	1
	Time spend per flower*	6	5.31	0.504	-	-	-	-	-	-	-	-	-	1	0.28	0.599	1	1.00	0.318	-	-	-	-	-	-
Pollen beetle adults	Number of visitors	6	13.28	**0.039**	2	69.43	**<0.001**	12	32.49	**<0.001**	-	-	-	1	177.58	**<0.001**	1	11.93	**<0.001**	1	13.33	**<0.001**	-	-	-
Flowering stage																									
Inflorescences	Number	6	1.76	0.941	1	11.84	**<0.001**	6	6.04	0.418	-	-	-	-	-	-	1	100.72	**<0.001**	1	82.23	**<0.001**	-	-	-
All pollinators	Number of visitors	6	7.07	0.315	1	19.59	**<0.001**	6	21.75	**0.001**	-	-	-	1	13.54	**<0.001**	1	25.21	**<0.001**	-	-	-	-	-	-
	Flowers visited in total	6	7.92	0.244	1	6.21	**0.013**	6	3.07	0.800	-	-	-	1	0.559	0.455	1	0	1	-	-	-	1	19.60	**<0.001**
Honeybees	Number of visitors	6	7.42	0.284	1	14.91	**<0.001**	6	16.48	**0.011**	-	-	-	1	10.90	**<0.001**	1	20.85	**<0.001**	-	-	-	-	-	-
	Flowers visited in total	6	16.59	**0.011**	1	1.93	0.165	6	3.19	0.785	-	-	-	-	-	-	1	0	1	-	-	-	-	-	-
	Flowers visited per visit	6	5.90	0.434	1	0.04	0.837	6	8.77	0.187	-	-	-	-	-	-	1	168.35	**<0.001**	-	-	-	-	-	-
	Time spend per plant	6	7.20	0.303	1	1.87	0.171	6	2.81	0.832	1	0.32	0.574	-	-	-	-	-	-	-	-	-	1	1.20	0.274
	Time spend per flower	6	1.39	0.967	1	0.256	0.613	6	15.54	**0.016**	-	-	-	1	6.19	**0.013**	-	-	-	-	-	-	1	37.28	**<0.001**
Bumblebees	Number of visitors	5	0.629	0.987	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Syrphid flies	Number of visitors	6	8.36	0.213	1	2.81	*0.094*	6	5.14	0.527	-	-	-	1	51.78	**<0.001**	-	-	-	-	-	-	-	-	-
	Flowers visited in total*	6	6.74	0.346	-	-	-	-	-	-	-	-	-	1	3.54	*0.060*	1	422.35	**<0.001**	-	-	-	-	-	-
	Flowers visited per visit*	6	28.88	**<0.001**	-	-	-	-	-	-	-	-	-	1	283.97	**<0.001**	-	-	-	-	-	-	1	4.71	**0.030**
	Time spend per plant*	6	17.40	**0.008**	-	-	-	-	-	-	1	0	1	1	5.34	**0.021**	-	-	-	-	-	-	-	-	-
	Time spend per flower*	6	11.32	*0.079*	-	-	-	-	-	-	1	0	1	1	3.10	*0.078*	1	0	1	-	-	-	-	-	-
Pollen beetle adults	Number of visitors	6	7.96	0.241	1	13.89	**<0.001**	6	6.19	0.402	-	-	-	1	97.06	**<0.001**	1	13.10	**<0.001**	1	22.10	**<0.001**	-	-	-

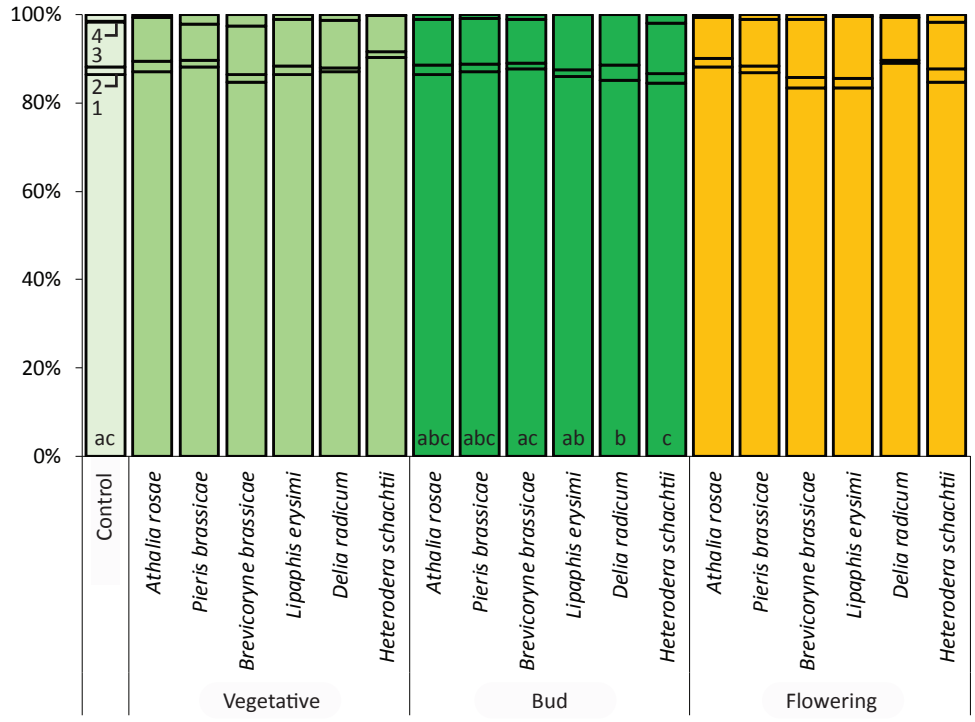


Fig. B1 Composition of pollinator communities of uninfested plots (control) of *Brassica nigra* plants and plots infested by herbivores at different plant ontogenetic stages. Communities consist of honeybees (1), bumblebees (2), syrphid flies (3), and solitary bees (4). Letters at the bottom of the bars indicate marginally insignificant differences for $0.05 < P < 0.1$ between herbivore species within a plant ontogenetic stage based on pairwise chi-square tests.

Table B5. Standardized residuals for the number of visitors of each pollinator group (honeybees, bumblebees, syrphid flies, and solitary bees) for uninfested *Brassica nigra* plants or plants infested with herbivores at different plant ontogenetic stages. Numbers displayed in bold exceed the ± 2 criteria.

	Uninfested (control)					
Honeybees	0.05					
Bumblebees	-0.12					
Syrphid flies	-0.48					
Solitary bees	1.36					
Vegetative	*Athalia rosae*	*Pieris brassicae*	*Brevicoryne brassicae*	*Lipaphis erysimi*	*Delia radicum*	*Heterodera schachtii*
Honeybees	0.55	1.05	-1.10	0.04	0.50	**2.02**
Bumblebees	0.72	-0.76	-0.13	0.01	-1.74	-0.74
Syrphid flies	-0.56	-1.47	0.22	-0.06	-0.02	-1.44
Solitary bees	-1.06	1.83	**3.09**	0.03	0.64	-1.38
Bud						
Honeybees	-0.02	0.39	0.94	-0.32	-0.88	-1.38
Bumblebees	0.52	0.04	-1.08	-0.56	**2.78**	0.69
Syrphid flies	-0.19	-0.23	-0.52	1.43	0.60	0.53
Solitary bees	-0.07	-0.66	-0.16	**-2.38**	**-2.43**	**2.04**
Flowering						
Honeybees	1.23	0.27	-1.99	-1.73	1.63	-1.05
Bumblebees	-0.11	-0.44	1.00	0.16	-1.97	1.75
Syrphid flies	-1.05	-0.14	1.71	**2.30**	-0.59	-0.07
Solitary bees	-0.76	0.10	0.17	-1.27	-1.03	1.35
Delia radicum	Vegetative	Bud	Flowering			
Honeybees	0.08	-1.54	1.48			
Bumblebees	-1.79	**3.88**	**-2.04**			
Syrphid flies	-0.02	0.71	-0.69			
Solitary bees	**2.45**	**-2.29**	-0.25			
Heterodera schachtii						
Honeybees	**2.52**	-1.32	-0.89			
Bumblebees	-1.31	-0.02	1.21			
Syrphid flies	-1.42	1.03	0.21			
Solitary bees	**-2.01**	1.26	0.54			

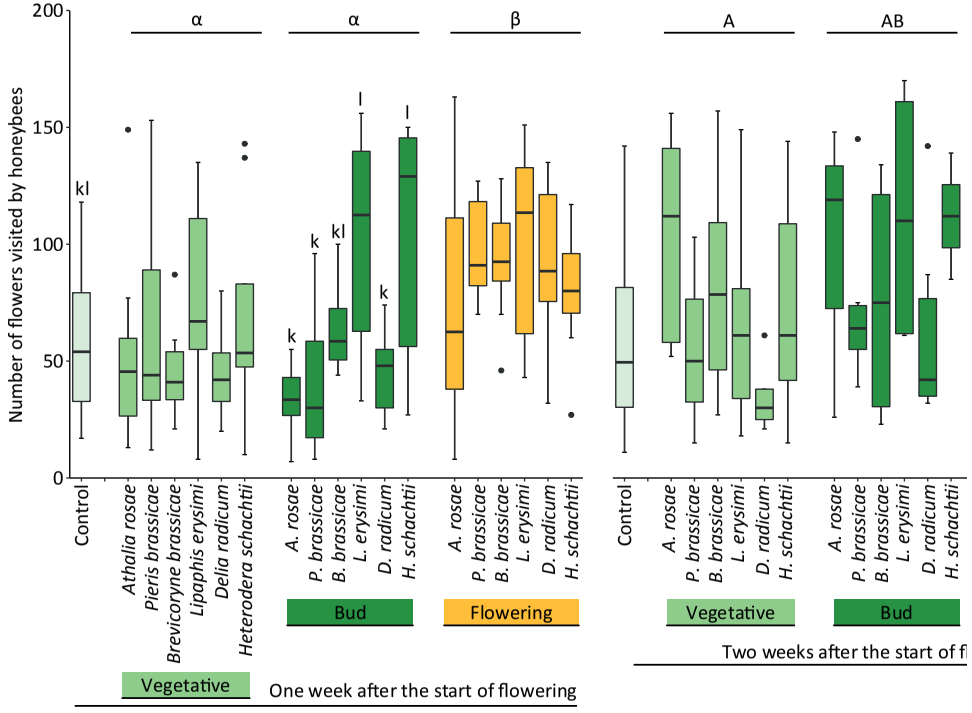


Fig. B2 Number of honeybee visits observed on flowers of uninfested plots (control) of *Brassica nigra* and on flowers of plots infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 - 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 7 and 9, and 16 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 10 for the control treatment. Letter groups (a - d, k - n, w - z) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests, and small or capital letters were used for different time-points. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

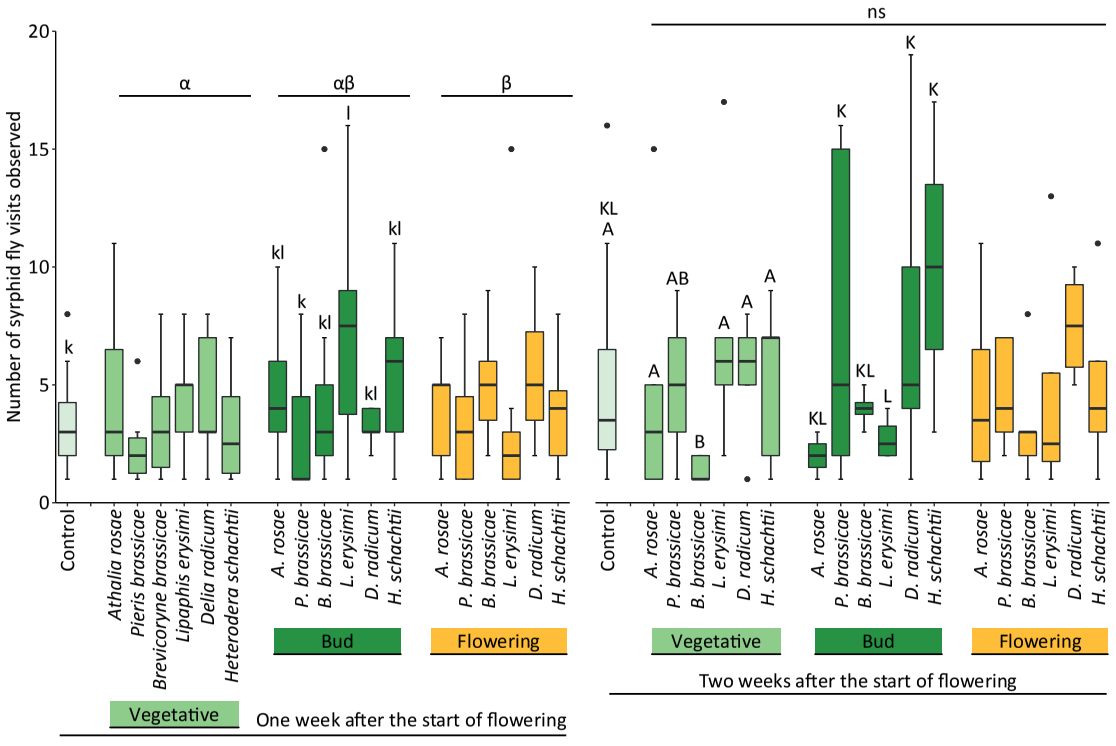


Fig. B3 Number of syrphid fly visits observed on flowers of uninfested plots (control) of *Brassica nigra* and on flowers of plots of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers are represented by circles (1.5 times the interquartile range below the 1st or above the 3rd quartile). Observations lasted for 10 min and were made at two time points: 7 - 9 and 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 5 and 8, and 12 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 5, and was 10 for the control treatment. Letter groups (a - d, k - n, w - z) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests, and small or capital letters were used for different time-points. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

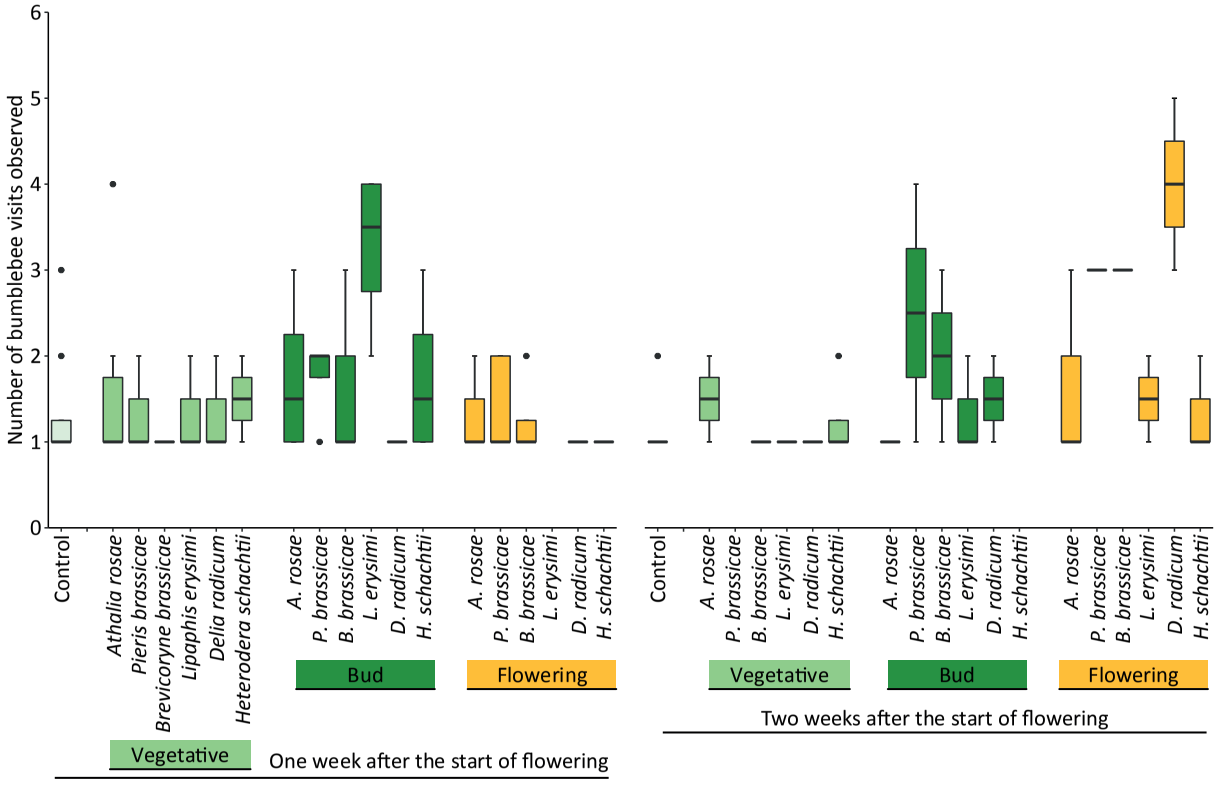


Fig. B4 Number of bumblebee visits observed on flowers of uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 - 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 0 and 6, and was 8 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 0 and 4, and was 5 for the control treatment.

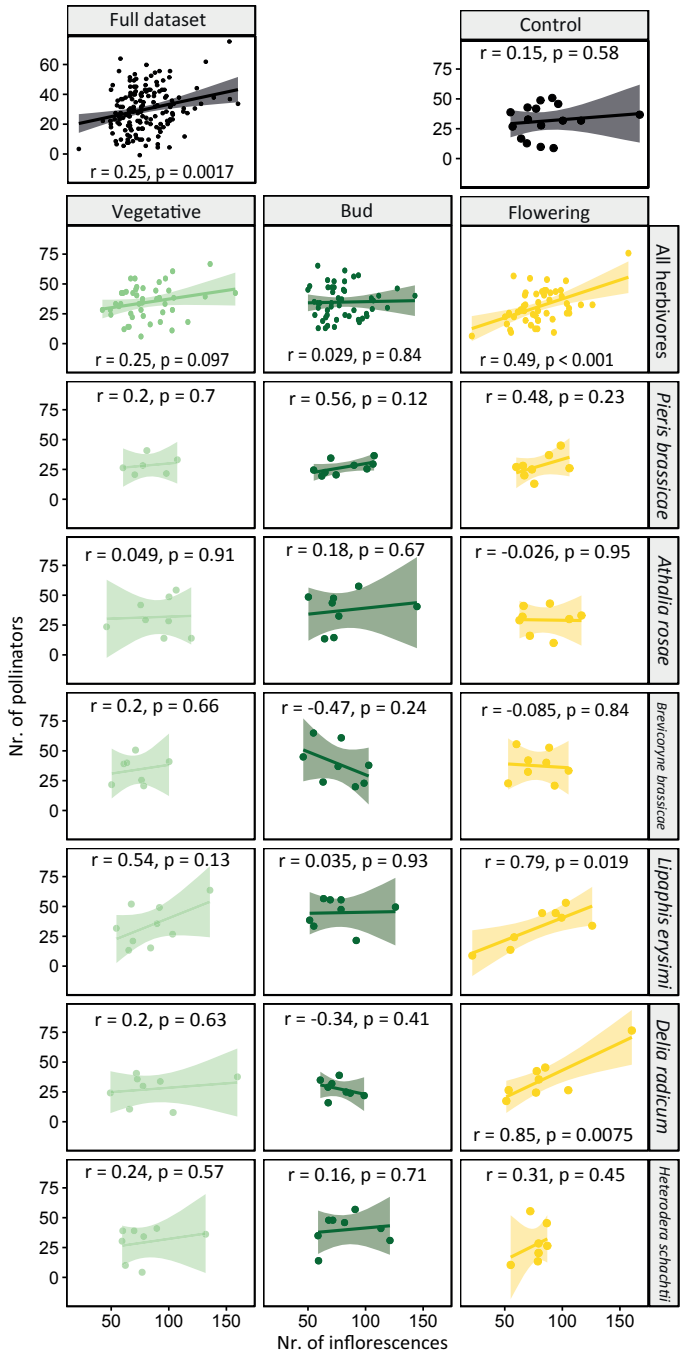


Fig. B5 Number of pollinator visits to plots of *Brassica nigra* with different numbers of inflorescences one week after plants in the plots had started flowering. Plots were uninfested (control) or infested with herbivores at different plant ontogenetic stages. Number of inflorescences per plot is the sum of 5 plants. Correlation coefficient r was computed using the Pearson method.

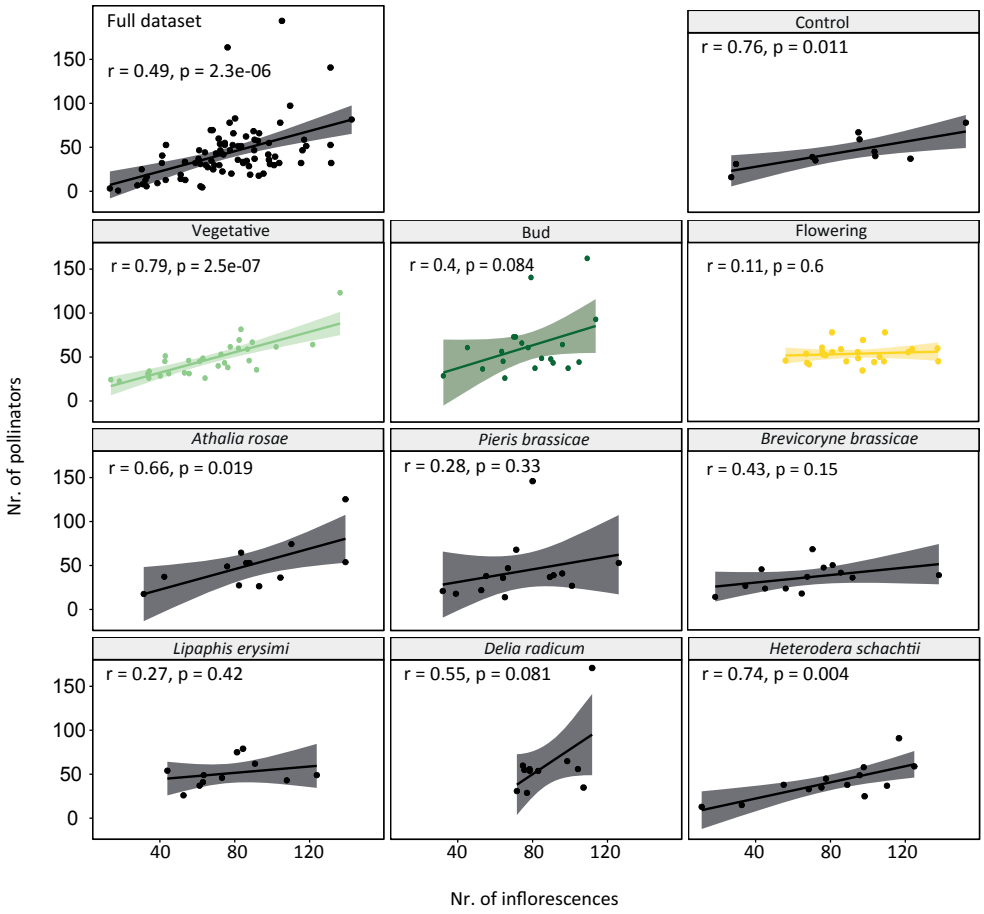


Fig. B6 Number of pollinators visits to plots of *Brassica nigra* with different numbers of inflorescences two weeks after plants in the plots had started flowering. Plots were uninfested (control) or infested with herbivores at different plant ontogenetic stages. Number of inflorescences per plot is the sum of 5 plants. Correlation coefficient r was computed using the Pearson method.

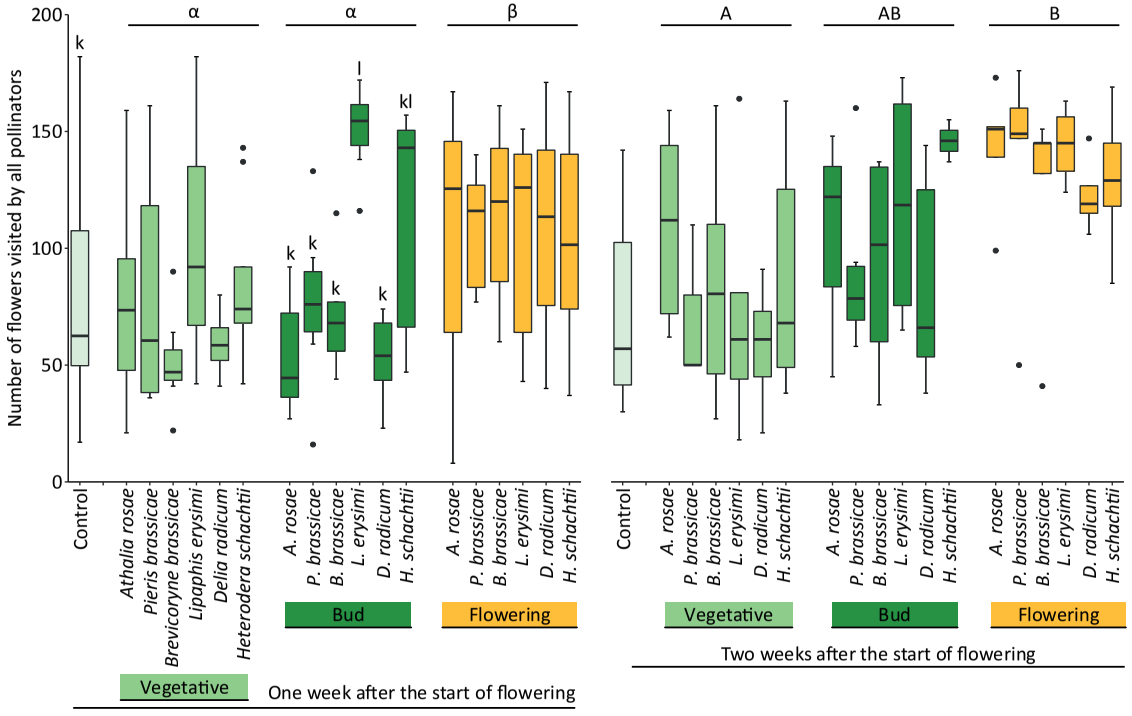


Fig. B7 Number of flowers visited by all pollinators of uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 - 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 7 and 9, and was 16 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 10 for the control treatment. Letters group (k - n) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, and small or capital letters were used for different time-points.

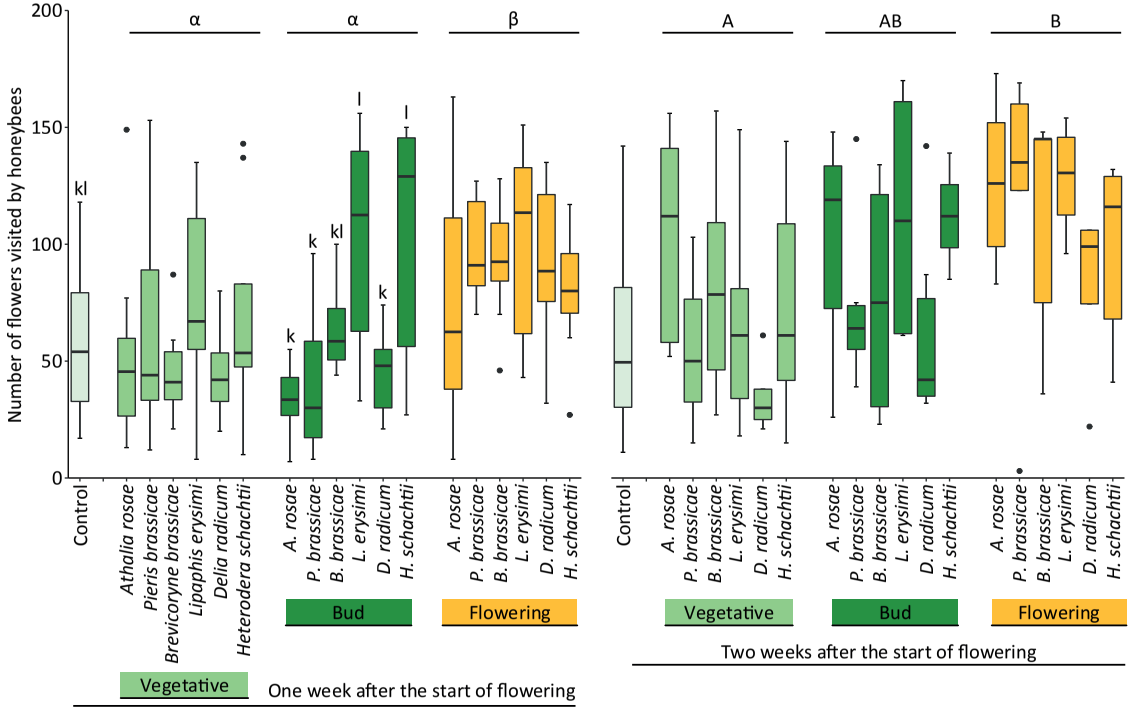


Fig. B8 Number of flowers visited by honeybees of uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 - 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 7 and 9, and was 16 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 10 for the control treatment. Letters group (k - n) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests and small or capital letters were used for different time-points.

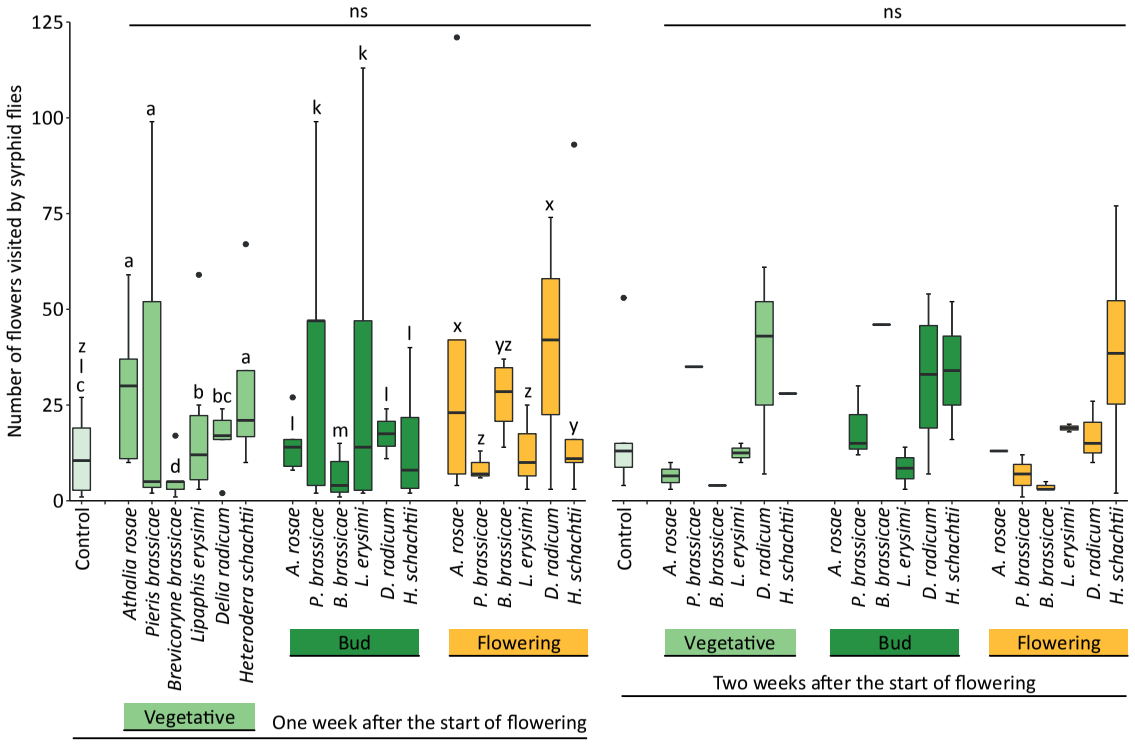


Fig. B9 Number of flowers visited syrphid flies of uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 – 9 days and between 14 and 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 8 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 0 and 4, and was 6 for the control treatment. Letter groups (a - d, k - n, w - z) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. *ns* indicates no differences between plant ontogenetic stages based on Tukey's *post hoc* tests.

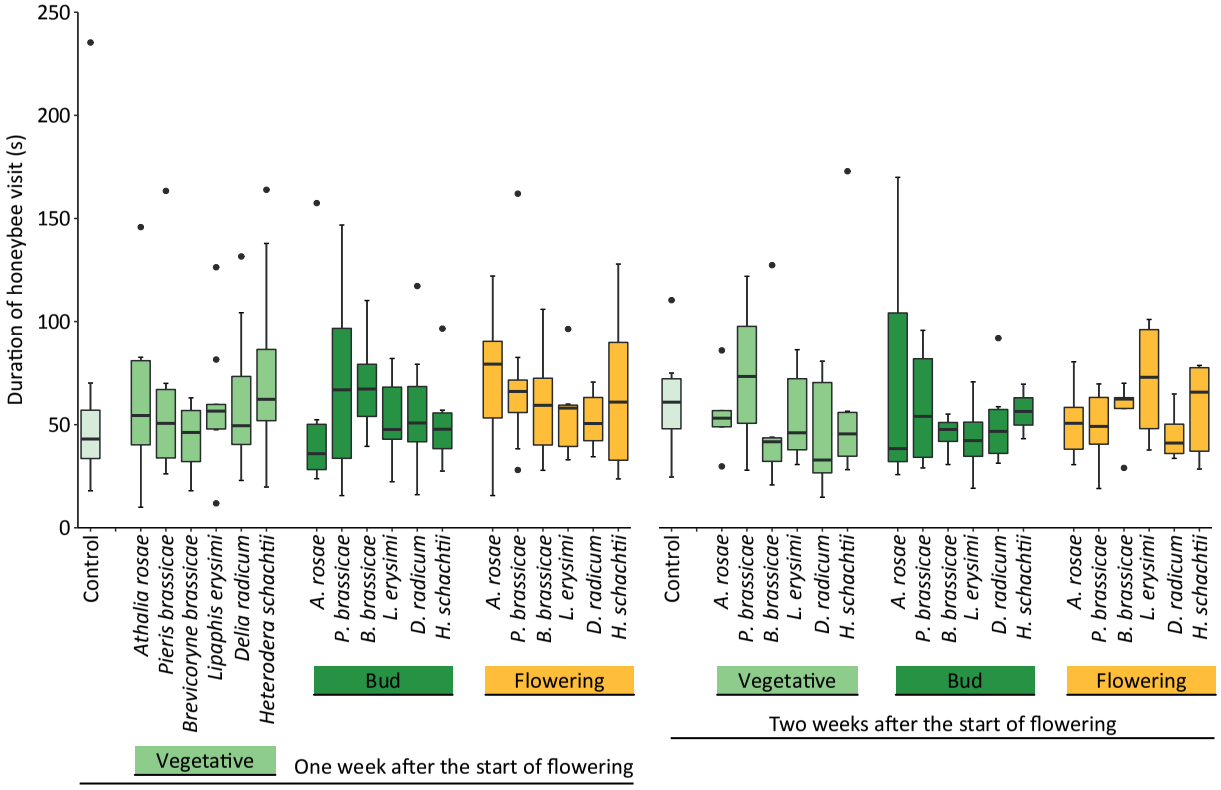


Fig. B10 Duration of honeybee visits to uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 – 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 7 and 9, and was 16 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 10 for the control treatment.

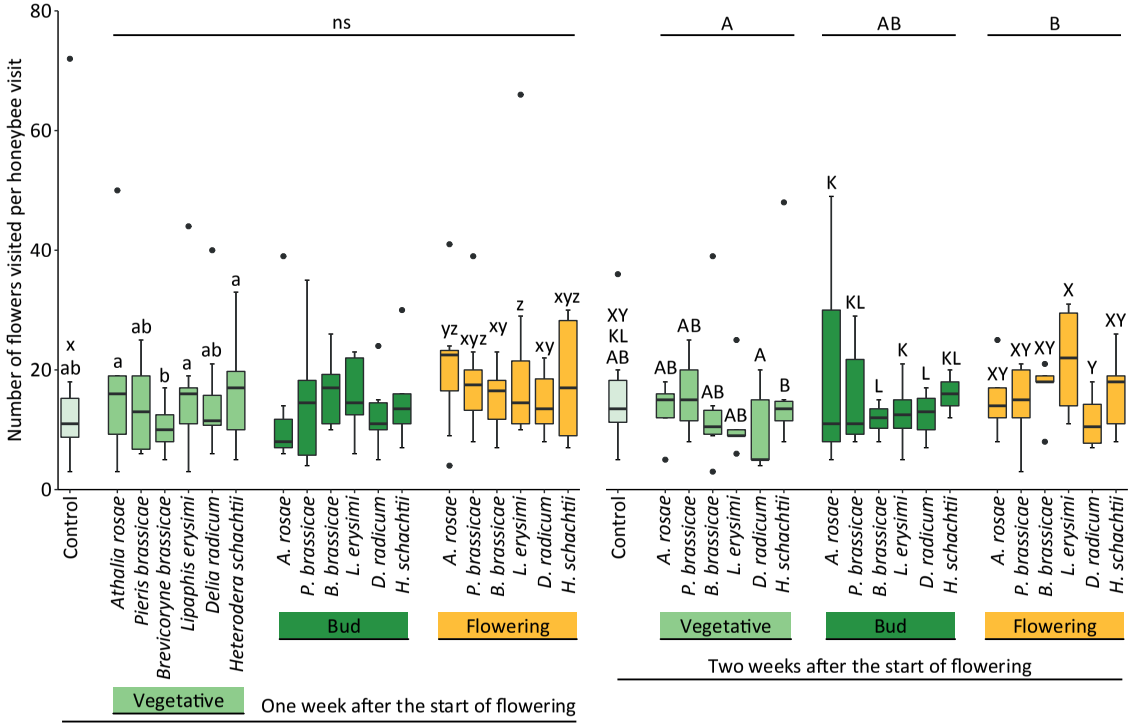


Fig. B11 Number of flowers visited per honeybee visit to uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 – 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 7 and 9, and was 16 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 10 for the control treatment. Letter groups (a - d, k - n, w - z) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests, and small or capital letters were used for different time-points. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

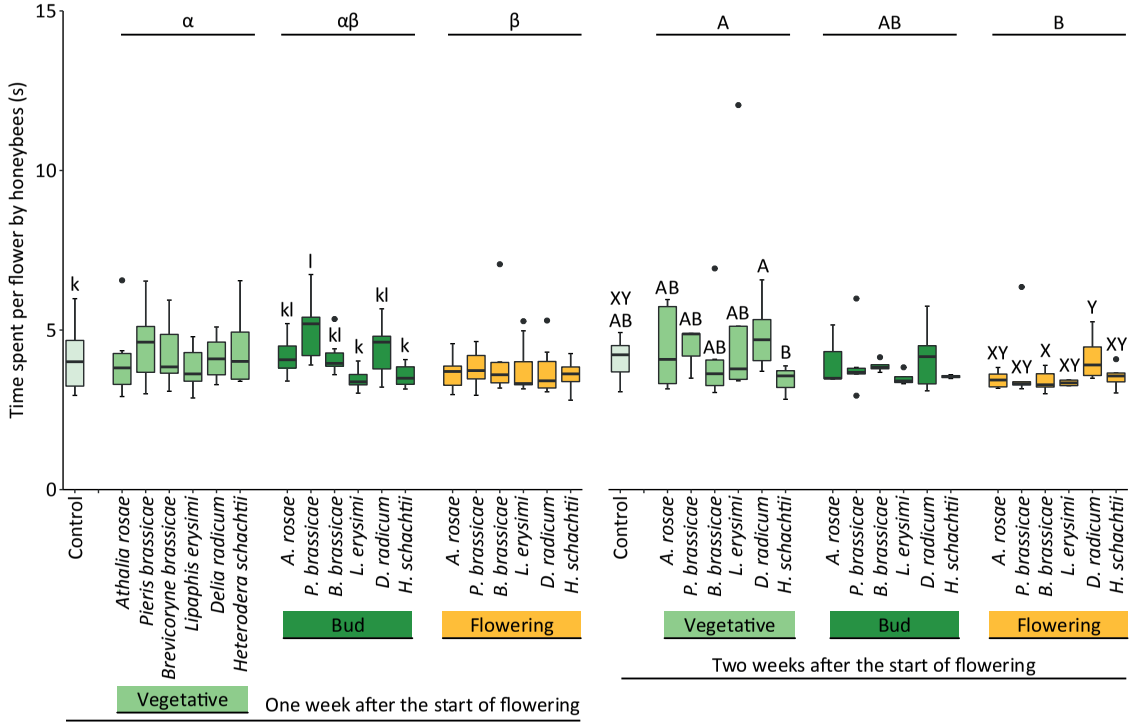


Fig. B12 Time spent per flower by honeybees of uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 - 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 7 and 9, and was 16 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 10 for the control treatment. Letter groups (a - d, k - n, w - z) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests, and small or capital letters were used for different time-points. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

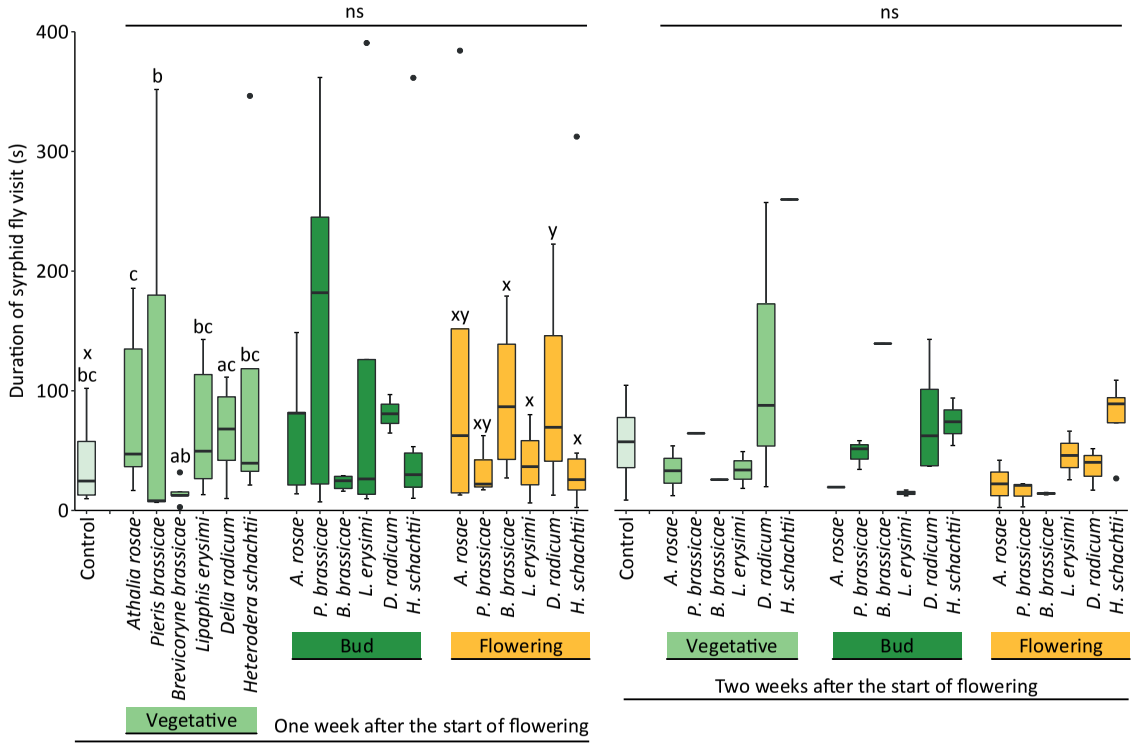


Fig. B13 Duration of syrphid fly visit to uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 – 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 8 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 0 and 4, and was 6 for the control treatment. Letter groups (a - d, w - z) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. *ns* indicates no differences between plant ontogenetic stages based on Tukey's *post hoc* tests.

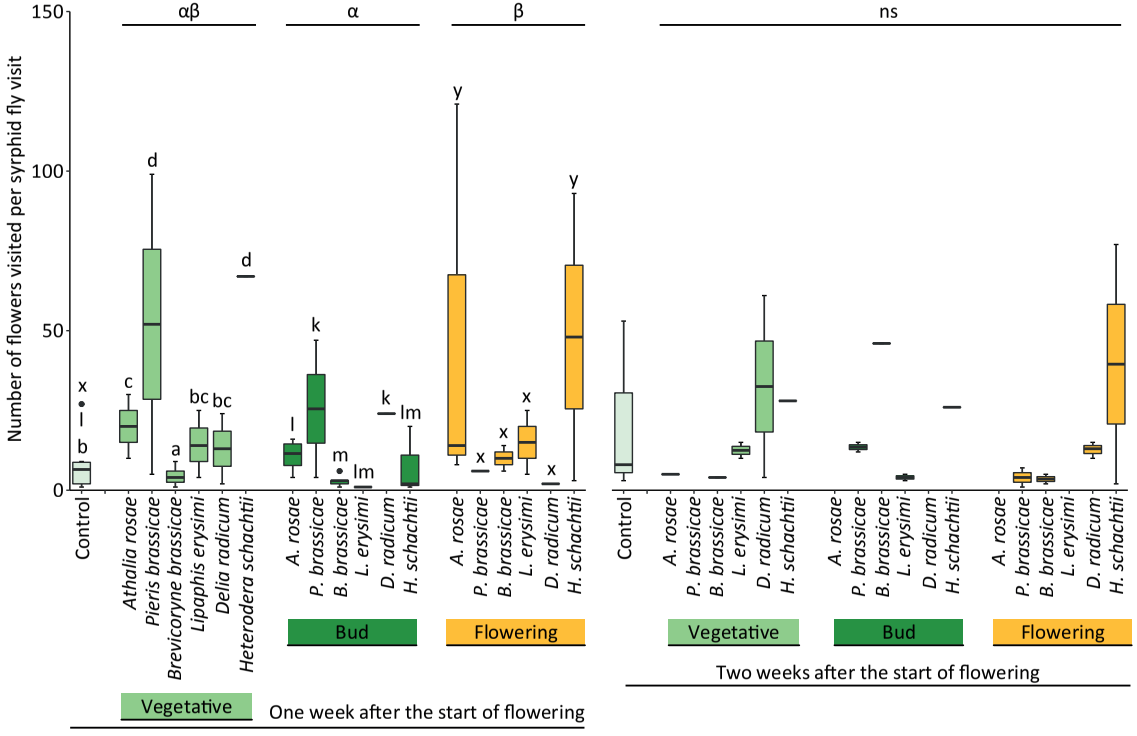


Fig. B14 Number of flowers visited per syrphid fly visit to uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 – 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 8 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 0 and 4, and was 6 for the control treatment. Letter groups (a - d, k - n, w - z) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

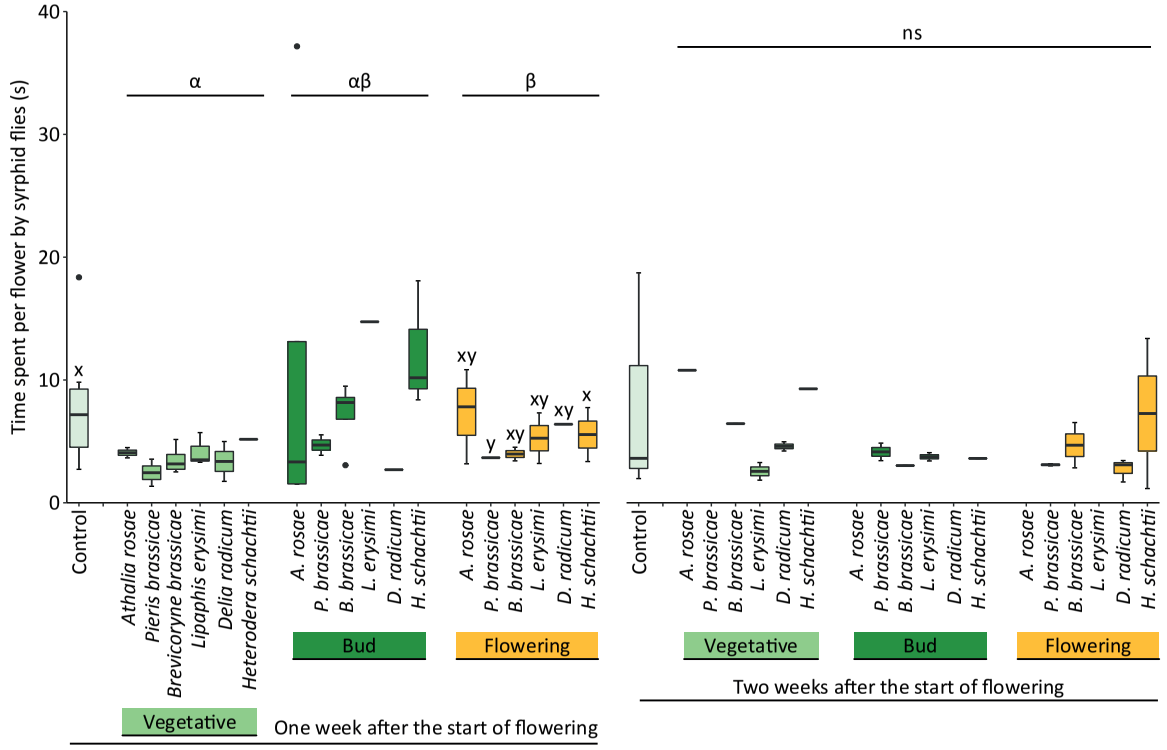


Fig. B15 Time spent per flower by syrphid flies of uninfested plots (control) of *Brassica nigra* and on flowers of plants infested by herbivores at different plant ontogenetic stages. Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Observations lasted for 10 min and were made at two time points: between 7 - 9 days and between 14 - 16 days after plots had started flowering. For 7 - 9 days after plots had started flowering, the number of replicates per herbivore treatment varied between 2 and 6, and was 8 for the control treatment. For 14 - 16 days after plots had started flowering, the number of replicates per herbivore treatment varied between 0 and 4, and was 6 for the control treatment. Letter group (w - z) above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.

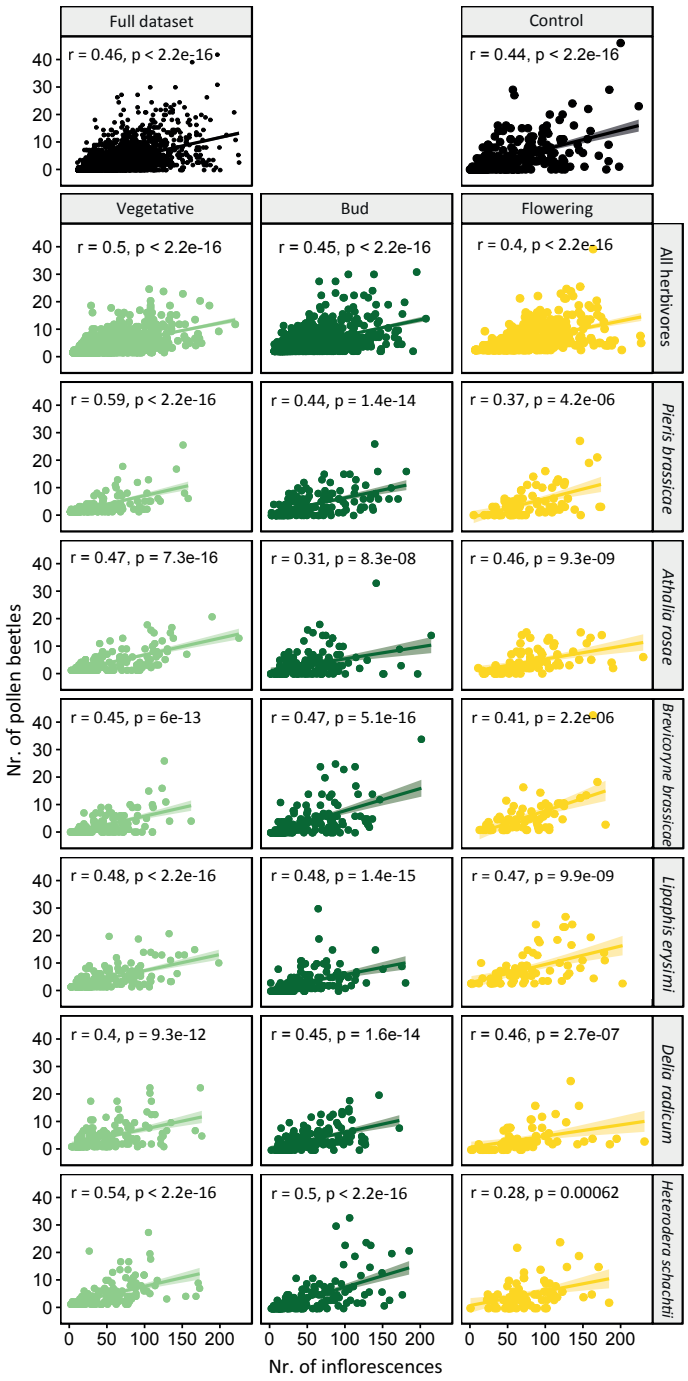


Fig. B16 Number of adult pollen beetles on flowers of *Brassica nigra* with different numbers of inflorescences one week after plots had reached the bud stage. Plots were uninfested (control) or infested with herbivores at different plant ontogenetic stages. Correlation coefficient r was computed using the Kendall method.

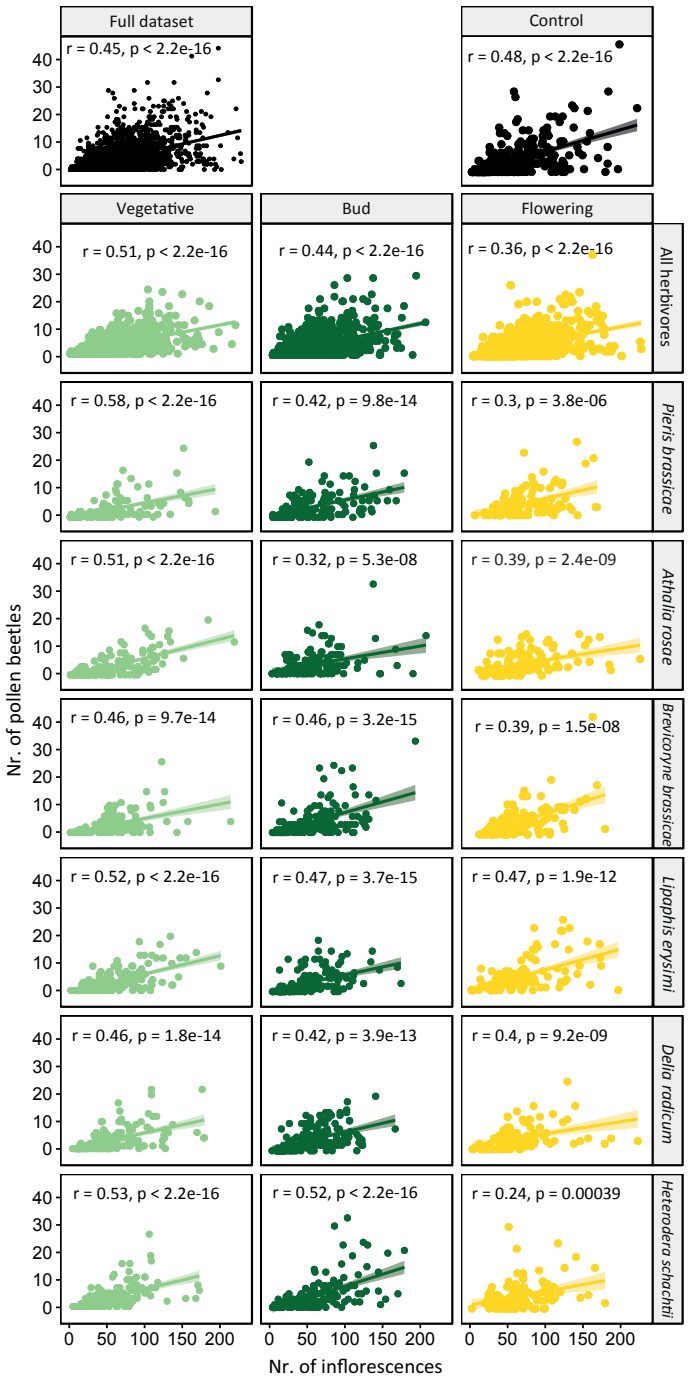


Fig. B17 Number of adult pollen beetles on flowers of *Brassica nigra* with different numbers of inflorescences one week after plots had started flowering. Plots were uninfested (control) or infested with herbivores at different plant ontogenetic stages. Correlation coefficient r was computed using the Kendall method.

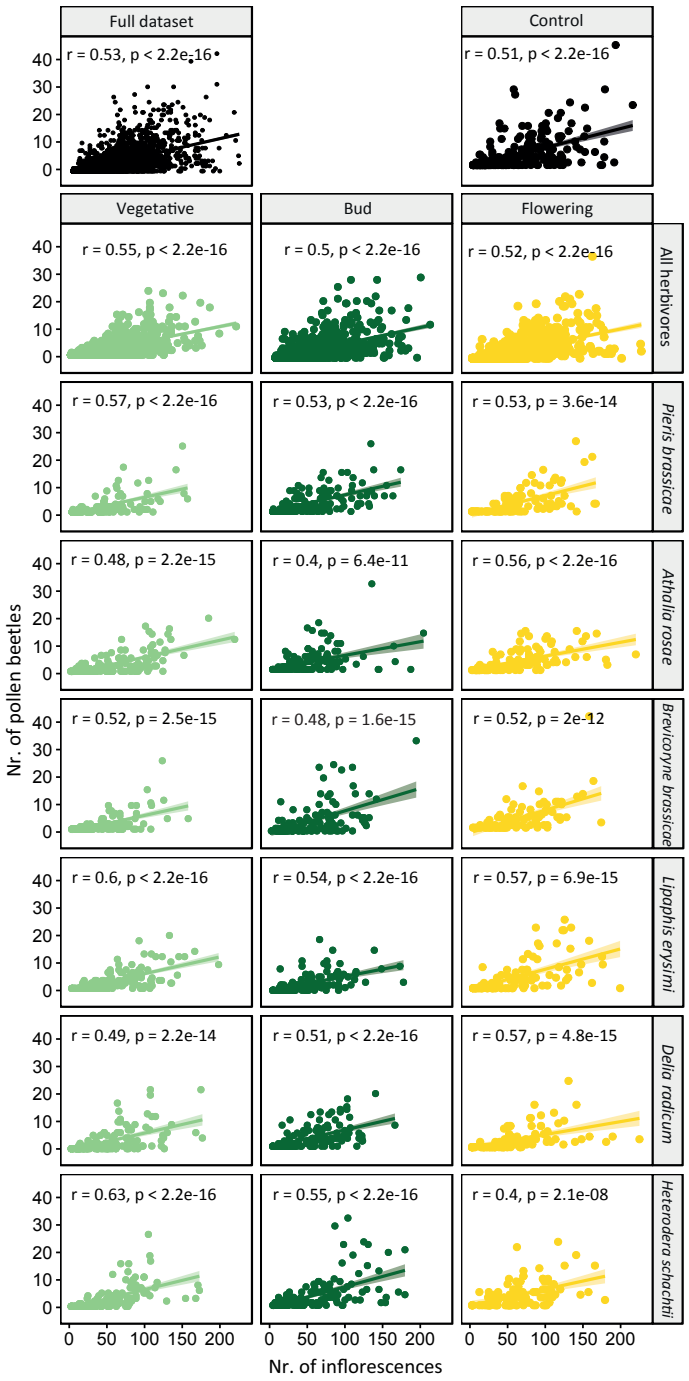


Fig. B18 Number of adult pollen beetles on flowers of *Brassica nigra* with different numbers of inflorescences two weeks after plots had started flowering. Plots were uninfested (control) or infested with herbivores at different plant ontogenetic stages. Correlation coefficient r was computed using the Kendall method.

Table B6. Output of generalized linear (mixed) models showing the effects of different fixed (herbivore species and plant ontogenetic stage) factors on plant seed set. All random factors were initially included in the model, and factors which explained less than 0.03 variation or with a *P* - value above 0.05 were excluded from the model. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

	Fixed factors									Random factors					
	Herbivore species (T)			Plant ontogenetic stage (O)			T*O			Plot			Plant position		
Number of seeds	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*	df	χ^2	*P*
per plot	5	15.14	**0.010**	2	3.49	0.175	10	28.72	**0.001**	1	7.18	**0.007**	1	26.23	**<0.001**
Vegetative stage	6	35.76	**<0.001**	-	-	**-**	-	-	**-**	1	12.45	**<0.001**	1	24.87	**<0.001**
Bud stage	6	0.386	0.999	-	-	**-**	-	-	**-**	1	0.89	0.347	1	6.96	**0.008**
Flowering stage	6	5.81	0.445	-	-	**-**	-	-	**-**	1	8.42	**0.004**	1	35.46	**<0.001**
per central plant	5	12.70	**0.026**	2	2.94	0.230	10	23.05	**0.011**	-	-	**-**	-	-	**-**
Vegetative stage	6	23.75	**<0.001**	-	-	**-**	-	-	**-**	-	-	**-**	-	-	**-**
Bud stage	6	6.93	0.327	-	-	**-**	-	-	**-**	-	-	**-**	-	-	**-**
Flowering stage	6	2.77	0.838	-	-	**-**	-	-	**-**	-	-	**-**	-	-	**-**
per side plant	5	11.83	**0.037**	2	8.51	**0.014**	10	19.39	**0.036**	1	4.08	**0.043**	-	-	**-**
Vegetative stage	6	24.54	**<0.001**	-	-	**-**	-	-	**-**	1	1.23	0.268	-	-	**-**
Bud stage	6	0.844	0.991	-	-	**-**	-	-	**-**	1	0	1	-	-	**-**
Flowering stage	6	11.84	*0.066*	-	-	**-**	-	-	**-**	1	1.45	**0.**229	-	-	**-**
Weight of 100 seeds	5	26.54	**<0.001**	2	9.14	**0.010**	10	29.78	**<0.001**	-	-	**-**	-	-	**-**
Vegetative stage	6	38.20	**<0.001**	-	-	**-**	-	-	**-**	-	-	**-**	-	-	**-**
Bud stage	6	8.60	0.197	-	-	**-**	-	-	**-**	-	-	**-**	-	-	**-**
Flowering stage	6	10.71	*0.098*	-	-	**-**	-	-	**-**	-	-	**-**	-	-	**-**

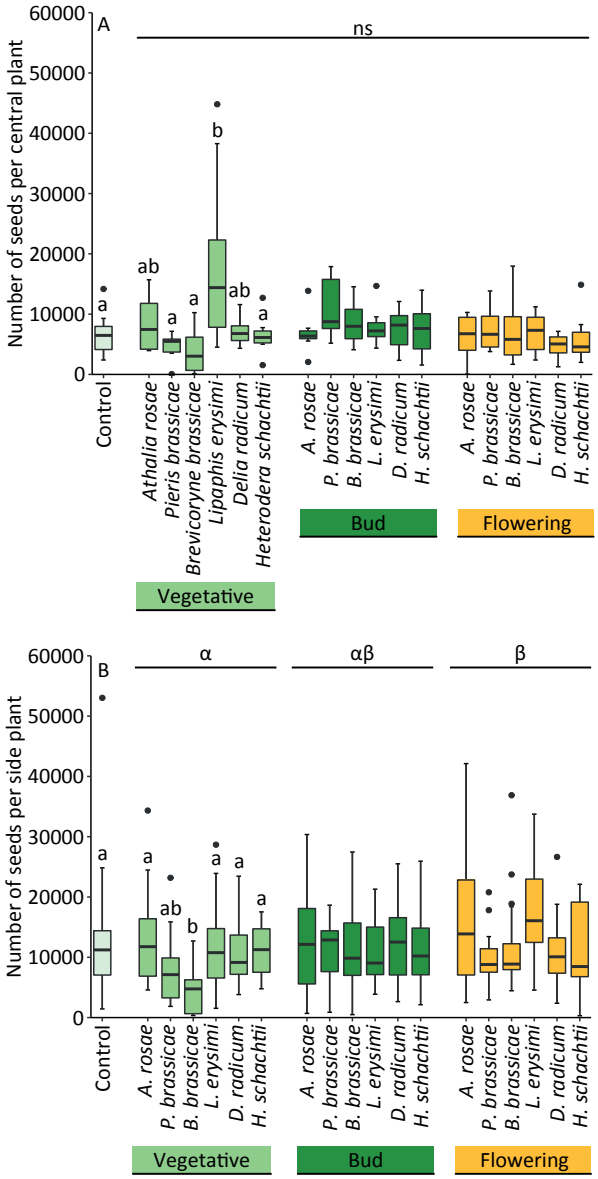
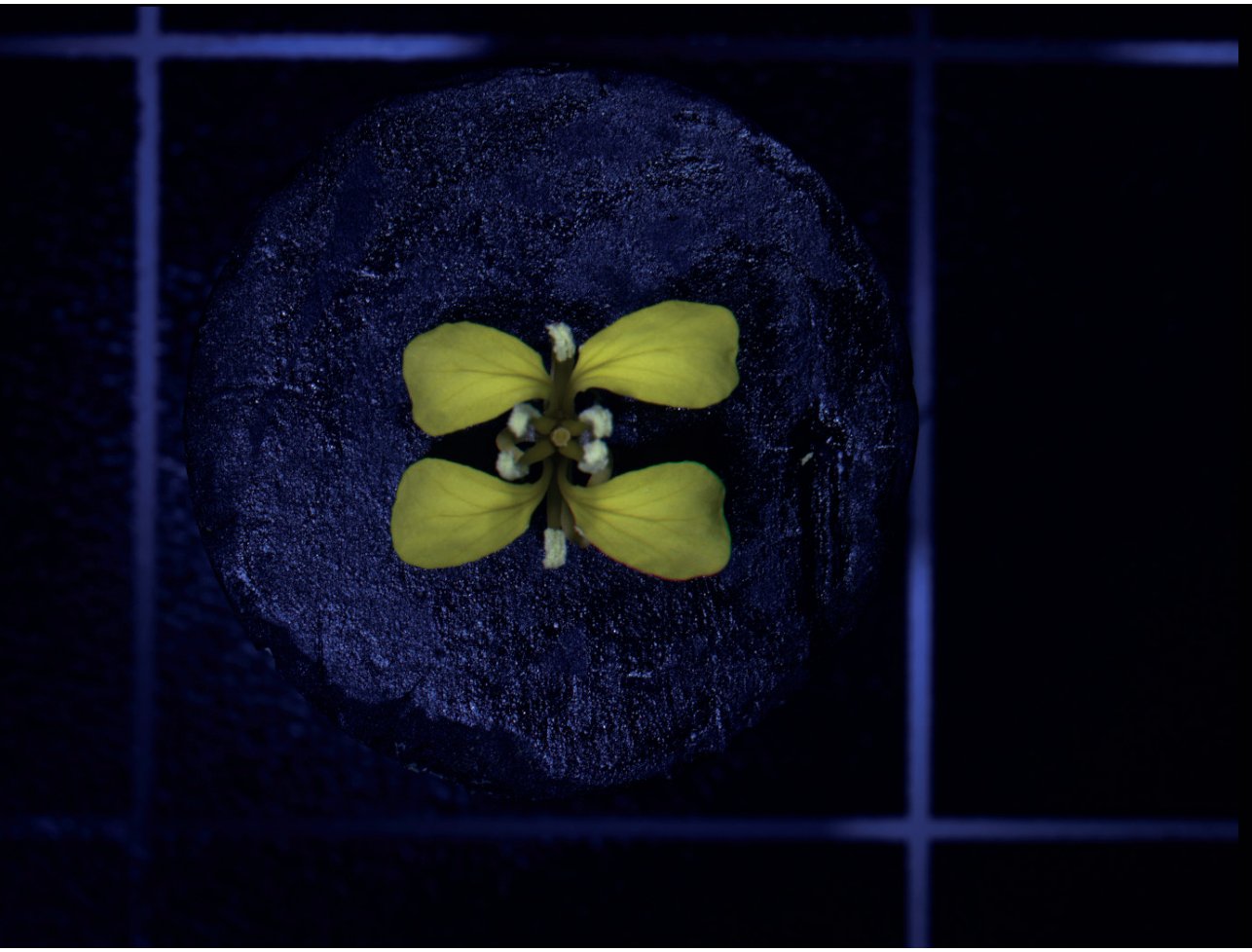


Fig. B19 Number of seeds produced by uninfested (control) *Brassica nigra* plants and plants infested by herbivores at different plant ontogenetic stages. We assessed the average number of seeds produced per central plant (A) and per side plant (B). Boxplots show median (line), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. For central plants, the number of replicates per herbivore treatment varied between 14 and 17, and was 28 for uninfested plants. For side plants, the number of replicates per herbivore treatment varied between 29 and 35, and was 63 for uninfested plants. Letters above bars indicate significant differences at ($P < 0.05$) between herbivore species within a plant ontogenetic stage based on Tukey's *post hoc* tests. Greek letters above lines indicate significant differences at ($P < 0.05$) between plant ontogenetic stages based on Tukey's *post hoc* tests, whereas *ns* indicates no differences.



Appendix C

Chapter 5
Floral plasticity: Herbivore-species-specific-induced changes in flower traits with contrasting effects on pollinator visitation.

Quint Rusman, Erik H. Poelman,
Farzana Nowrin, Gerrit Polder, and Dani Lucas-Barbosa.

Plant, Cell & Environment, *In press*

Table C1. Specifications of the eight filters of the multispectral camera used to photograph flowers of herbivore-infested and uninfested *Brassica nigra* plants.

Filter	Wavelength [nm]	Bandwidth [nm]	Exposure [ms]	Gain [dB]
1	425	50	20	100
2	466	21	30	0
3	500	20	10	0
4	542	10	7	0
5	570	50	1	0
6	601	13	3	0
7	640	20	3	0
8	680	20	3	0

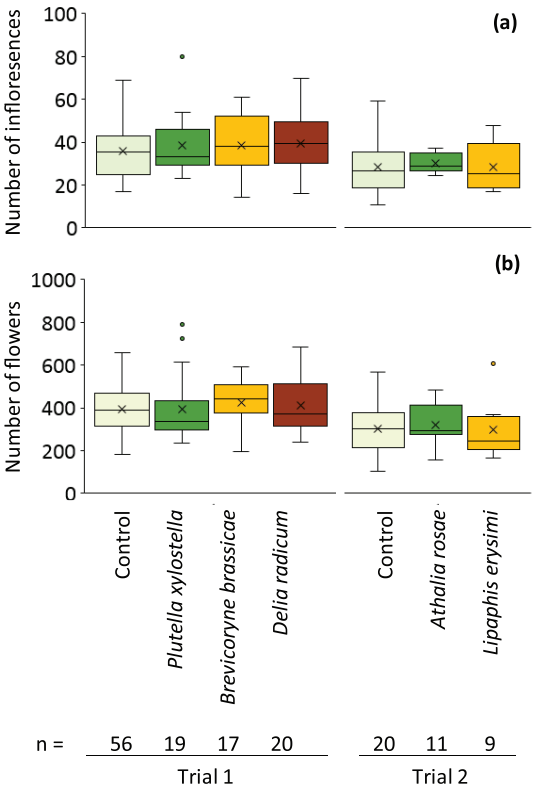


Fig. C1 Number of flowers (a) and inflorescences (b) of *Brassica nigra* plants infested with different herbivores or uninfested plants. Boxplots show median (line), mean (x), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Flowers and inflorescences were counted after seven days of herbivory.

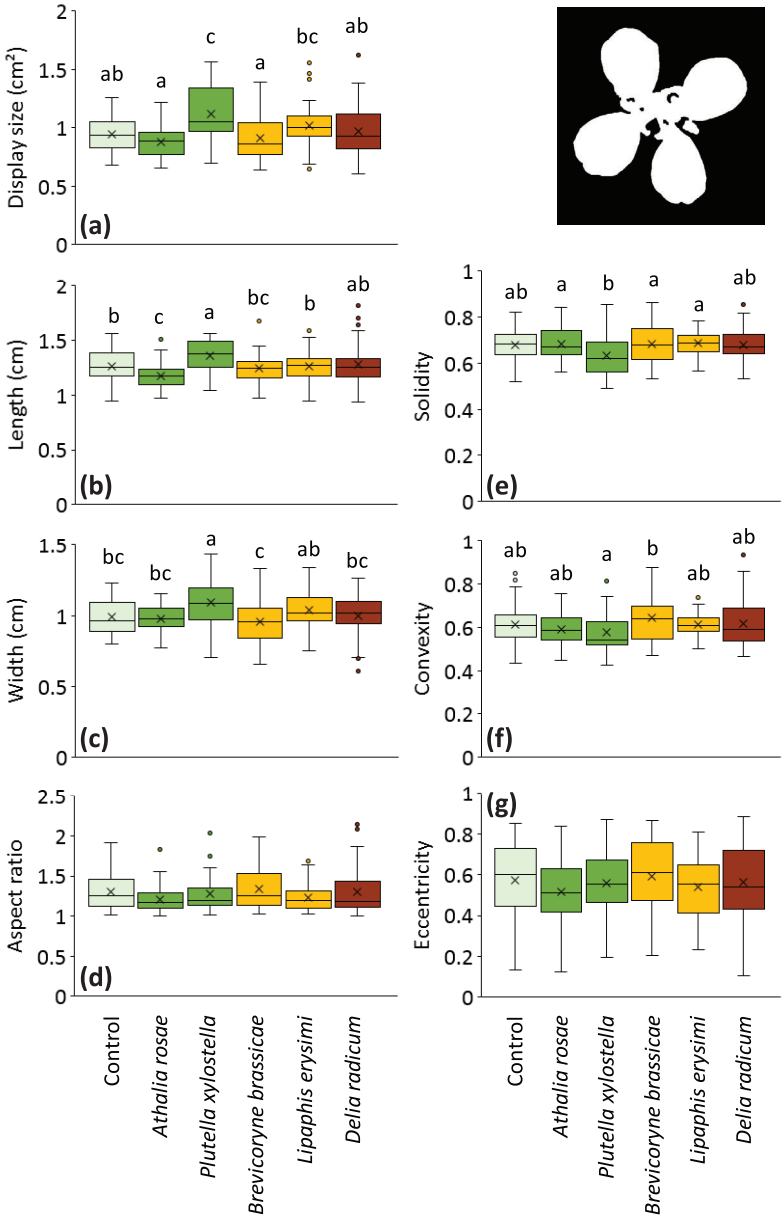


Fig. C2 Morphometry for flowers of uninfested *Brassica nigra* plants and plants infested with different herbivores. We measured a) display size, b) length, c) width, d) aspect ratio, e) solidity, f) convexity, and g) eccentricity. Boxplots show median (line), mean (x), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Measurements were taken after seven days of herbivory. Number of replicates per herbivore treatment varied between seven and eight plants, and six flowers were measured from each plant. Letters above bars indicate significant differences at $\alpha = 0.05$ based on Tukey's *post hoc* tests.

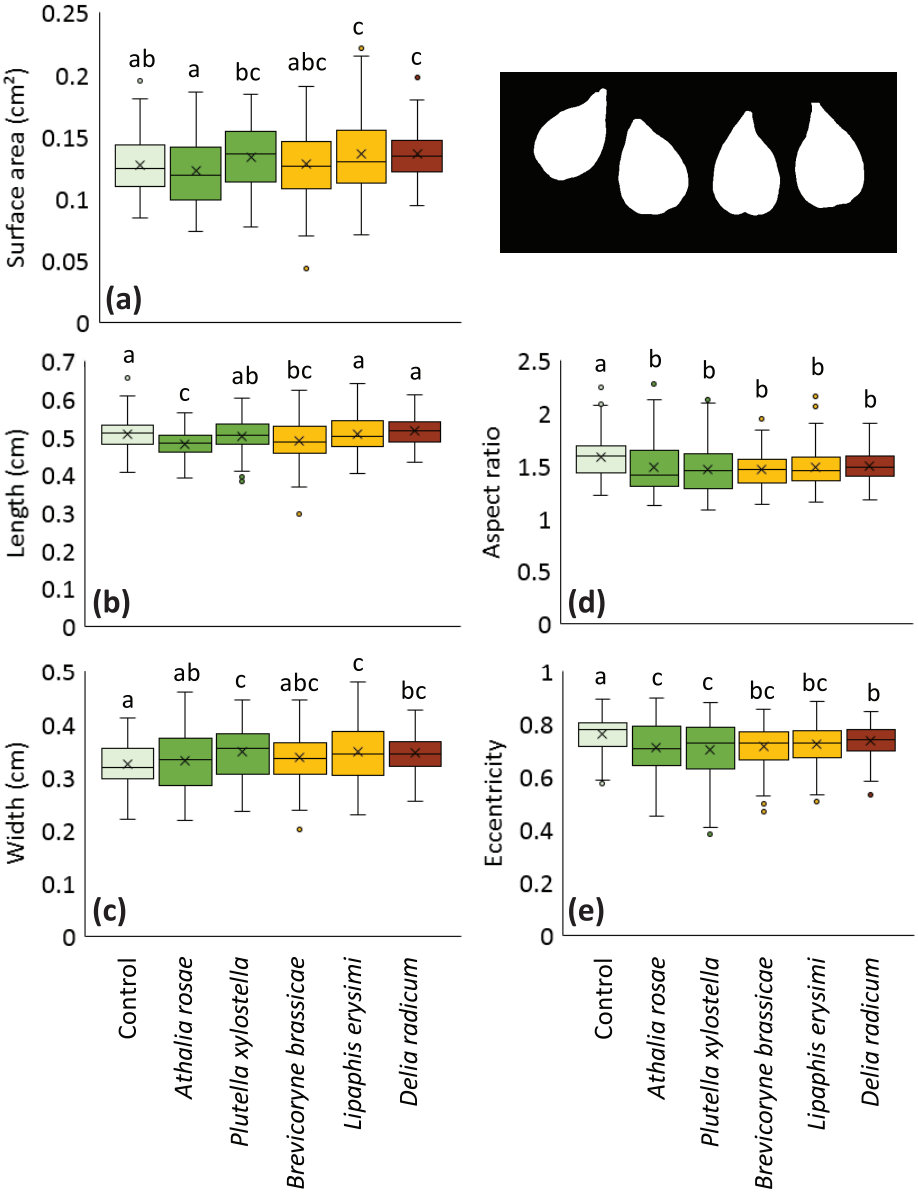


Fig. C3 Morphometry for petals of uninfested *Brassica nigra* plants and plants infested with different herbivores. We measured a) surface area, b) major chord length, c) minor chord length, d) aspect ratio, and e) eccentricity. Boxplots show median (line), mean (x), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Measurements were taken after seven days of herbivory, by taking pictures of all petals from each flower and subsequent software processing. Number of replicates per herbivore treatment varied between seven and eight plants, six flowers were used from each plant. Letters above bars indicate significant differences at $\alpha = 0.05$ based on Tukey's *post hoc* tests.

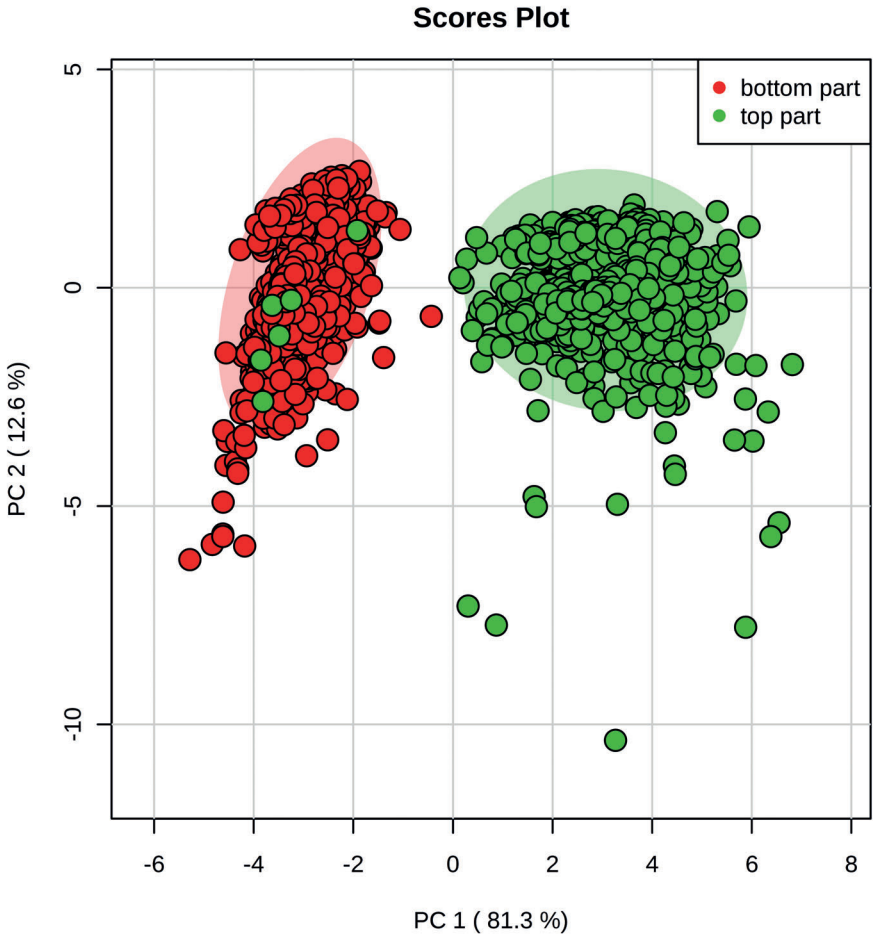


Fig. C4 Principal component analysis of the color profile of the top (green) and base (red) part of petals of *Brassica nigra* flowers. Highlighted areas denote 95% confidence intervals. Reflectance spectra measurements were done after seven days of herbivory. In total, the top and base parts of 1080 petals were measured, from 270 flowers from 45 plants.

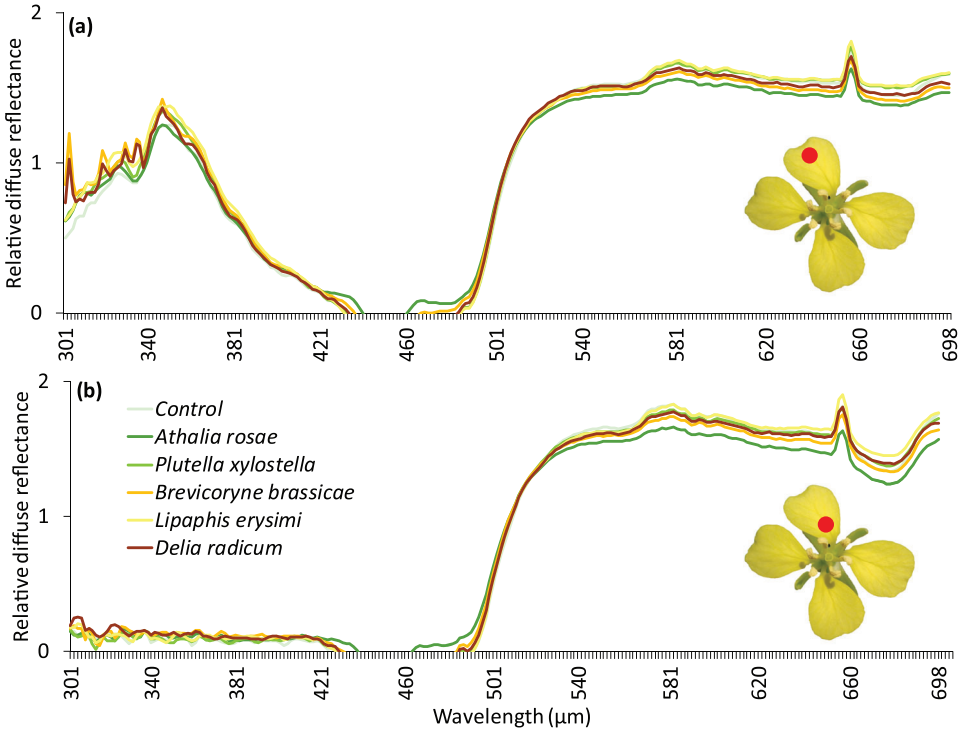


Fig. C5 Reflectance spectra with relative diffuse reflectance of wavelengths (300-700 nm) of the top (a) and base (b) part of petals of *Brassica nigra* plants infested with different herbivores or uninfested plants. The flower with the red dot indicates where measurements were taken (top or base), which was done after seven days of herbivory. Number of replicates per herbivore treatment varied between six and eight plants, and from each plant, all four petals of six flowers were measured.

Table C2. Confusion matrices of support vector machine classifiers for the reflectance spectra of top parts of petals of uninfested *Brassica nigra* plants or plants infested with different herbivores (a) or herbivore functional groups (HFGs) (b). Herbivore functional groups were assigned as followed: Chewing herbivores: *Athalia rosae* and *Plutella xylostella*; sap-feeding herbivores: *Brevicoryne brassicae* and *Lipaphis erysimi*; root herbivores: *Delia radicum*. The models assigned individual herbivore species to the correct HFGs (c) and *vice versa* (d) based on the reflectance spectra. The flower with the red dot indicates where measurements were taken (top or base), which was done after seven days of herbivory. Number of replicates per herbivore treatment varied between six and eight plants, and from each plant, all four petals of six flowers were measured.

(a)

Predicted by SVM model \ True label	Control	*Athalia rosae*	*Plutella xylostella*	*Brevicoryne brassicae*	*Lipaphis erysimi*	*Delia radicum*
Control	57	1	0	0	1	0
Athalia rosae	0	41	7	0	0	0
Plutella xylostella	0	4	43	1	0	0
Brevicoryne brassicae	0	0	1	31	6	0
Lipaphis erysimi	1	0	0	8	42	0
Delia radicum	0	0	0	0	1	43

Accuracy	89 %
Error rate	10 %

(b)

Predicted by SVM model \ True label	Control	Chewing herbivores	Sap-feeding herbivores	Root herbivores
Control	56	0	1	0
Chewing herbivores	1	96	1	0
Sap-feeding herbivores	1	1	87	0
Root herbivores	0	0	0	43

Accuracy	98 %
Error rate	2 %

(c)

Predicted by SVM model \ True label	Control	*Athalia rosae*	*Plutella xylostella*	*Brevicoryne brassicae*	*Lipaphis erysimi*	*Delia radicum*
Control	56	0	0	0	1	0
Chewing herbivores	1	46	50	1	0	0
Sap-feeding herbivores	1	0	1	39	48	0
Root herbivores	0	0	0	0	0	43

Accuracy	97 %

(d)

Predicted by SVM model \ True label	Control	Chewing herbivores	Sap-feeding herbivores	Root herbivores
Control	57	1	1	0
Athalia rosae	0	48	0	0
Plutella xylostella	0	47	1	0
Brevicoryne brassicae	0	1	38	0
Lipaphis erysimi	1	0	49	0
Delia radicum	0	0	0	43

Accuracy	91 %

Table C3. Confusion matrices of support vector machine classifiers for the reflectance spectra of base parts of petals of uninfested *Brassica nigra* plants or plants infested with different herbivores (a) or herbivore functional groups (HFGs) (b). Herbivore functional groups were assigned as followed: Chewing herbivores: *Athalia rosae* and *Plutella xylostella*; sap-feeding herbivores: *Brevicoryne brassicae* and *Lipaphis erysimi*; root herbivores: *Delia radicum*. The models assigned individual herbivore species to the correct HFGs (c) and *vice versa* (d) based on the reflectance spectra. The flower with the red dot indicates where measurements were taken (top or base), which was done after seven days of herbivory. Number of replicates per herbivore treatment varied between six and eight plants, and from each plant, all four petals of six flowers were measured.

(a)

Predicted by SVM model \ True label	Control	*Athalia rosae*	*Plutella xylostella*	*Brevicoryne brassicae*	*Lipaphis erysimi*	*Delia radicum*
Control	63	0	1	0	2	0
Athalia rosae	0	38	8	0	0	0
Plutella xylostella	0	8	39	0	0	0
Brevicoryne brassicae	1	0	2	31	5	0
Lipaphis erysimi	1	0	0	4	43	2
Delia radicum	0	0	0	0	0	38

Accuracy	83 %
Error rate	12 %

(b)

Predicted by SVM model \ True label	Control	Chewing herbivores	Sap-feeding herbivores	Root herbivores
Control	64	0	1	0
Chewing herbivores	0	92	0	0
Sap-feeding herbivores	1	4	84	2
Root herbivores	0	0	0	38

Accuracy	92 %
Error rate	4 %

(c)

Predicted by SVM model \ True label	Control	*Athalia rosae*	*Plutella xylostella*	*Brevicoryne brassicae*	*Lipaphis erysimi*	*Delia radicum*
Control	64	0	0	0	1	0
Chewing herbivores	0	46	47	0	0	0
Sap-feeding herbivores	1	0	4	35	49	2
Root herbivores	0	0	0	0	0	38

Accuracy	93 %

(d)

Predicted by SVM model \ True label	Control	Chewing herbivores	Sap-feeding herbivores	Root herbivores
Control	63	1	2	0
Athalia rosae	0	46	0	0
Plutella xylostella	0	47	0	0
Brevicoryne brassicae	1	2	36	0
Lipaphis erysimi	1	0	47	2
Delia radicum	0	0	0	38

Accuracy	84 %

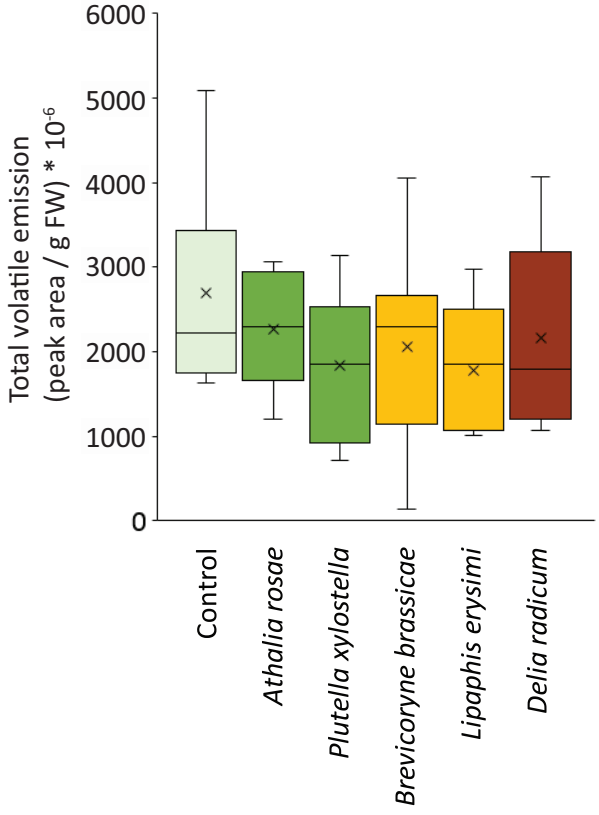


Fig. C6 Total volatile emission (peak area/ g FW) of uninfested flowering *Brassica nigra* plants and plants infested with different herbivores. Boxplots show median (line), mean (x), 1st and 3rd quartiles, minimum and maximum. Volatiles were collected after seven days of herbivory. Number of replicates per herbivore treatment varied between seven and nine plants.

Table C4. Volatile compounds of uninfested flowering *Brassica nigra* plants or plants infested with different herbivores. Volatiles were collected after seven days of herbivory. Peak area of volatile emission for each compound was corrected by g FW and divided by 10^5. Number of replicates per herbivore treatment varied between seven and nine plants.

Putatively identified volatile compounds	Arithmetic Index*	Uninfested control	*Athalia rosae*	*Plutella xylostella*	*Brevicoryne brassicae*	*Lipaphis erysimi*	*Delia radicum*
		Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD
Benzenoids and phenylpropanoids							
Benzaldehyde	**971**	3529 ± 2601	2181 ± 765	2334 ± 1368	1940 ± 954	2198 ± 1311	2345 ± 1032
Benzyl alcohol	**1039**	1048 ± 1228	787 ± 339	607 ± 417	615 ± 435	782 ± 532	898 ± 383
Phenylacetaldehyde	**1053**	2848 ± 3490	1829 ± 1690	1265 ± 1402	1664 ± 979	1377 ± 1402	1328 ± 1294
Benzyl acetate	**1148**	39 ± 62	23 ± 18	18 ± 17	20 ± 12	26 ± 27	28 ± 21
Methyl salicylate	**1207**	45 ± 97	10 ± 5	17 ± 29	31 ± 55	12 ± 11	15 ± 9
p-Anisaldehyde	**1269**	2227 ± 2418	1357 ± 631	1597 ± 1137	1074 ± 606	1106 ± 1099	1152 ± 734
Monoterpenoids							
α-Thujene	**932**	810 ± 588	662 ± 329	390 ± 290	569 ± 458	435 ± 350	568 ± 454
α-Pinene	**943**	1423 ± 556	1520 ± 373	1274 ± 827	1222 ± 618	1256 ± 454	1368 ± 409
Camphene	**960**	549 ± 622	332 ± 301	193 ± 161	429 ± 404	212 ± 188	297 ± 361
Sabinene	**981**	889 ± 428	807 ± 361	501 ± 367	643 ± 424	568 ± 330	679 ± 467
β-Pinene	**989**	286 ± 145	236 ± 132	176 ± 126	200 ± 143	172 ± 104	216 ± 172
β-Myrcene	**991**	382 ± 257	329 ± 121	193 ± 128	238 ± 159	246 ± 153	255 ± 172
α-Phellandrene	**1015**	212 ± 147	178 ± 94	95 ± 76	145 ± 119	118 ± 100	146 ± 114
α-Terpinene	**1025**	119 ± 173	64 ± 56	36 ± 38	67 ± 75	35 ± 31	59 ± 68
p-Cymene	**1033**	2 ± 2	1 ± 1	1 ± 1	1 ± 1	1 ± 1	1 ± 1
β-Ocimene, (*E*)-	**1050**	979 ± 566	623 ± 630	323 ± 430	708 ± 632	409 ± 562	641 ± 731
γ-Terpinene	**1065**	66 ± 72	45 ± 33	26 ± 20	41 ± 38	28 ± 22	39 ± 39
Terpinolene	**1094**	276 ± 279	195 ± 127	102 ± 87	168 ± 150	120 ± 89	168 ± 156
α-Pinene oxide	**1115**	21 ± 20	27 ± 28	9 ± 9	25 ± 35	20 ± 24	12 ± 9
Alloocimene, neo	**1131**	1319 ± 603	1061 ± 490	839 ± 610	935 ± 678	774 ± 415	1028 ± 678
2,6-Dimethyl-1,3,5,7-octatetraene, (*Z*)-	**1136**	53 ± 39	30 ± 29	32 ± 45	40 ± 36	22 ± 31	34 ± 34
β-Ocimene epoxide, (*E*)-	**1142**	32 ± 11	14 ± 9	22 ± 18	42 ± 59	17 ± 12	27 ± 22
Verbenol, (*E*)	**1158**	57 ± 27	38 ± 29	22 ± 25	40 ± 35	21 ± 18	26 ± 15
Pinocarvone	**1178**	76 ± 67	51 ± 37	35 ± 41	54 ± 43	32 ± 24	39 ± 37
α-Terpineol	**1207**	143 ± 122	87 ± 58	61 ± 56	64 ± 43	57 ± 35	78 ± 51
Myrtenal	**1211**	96 ± 84	64 ± 42	44 ± 49	66 ± 53	39 ± 26	52 ± 48
Verbenone	**1225**	514 ± 253	540 ± 231	203 ± 182	294 ± 218	402 ± 387	532 ± 326
Homoterpenoids							
4,8-Dimethyl-1,3,7-nonatriene, (*E*)-	**1115**	303 ± 310	458 ± 243	435 ± 459	266 ± 122	197 ± 133	518 ± 675
4,8,12-Trimethyltrideca-1,3,7,11-tetraene , (*E, E*)-	**1578**	34 ± 17	93 ± 118	174 ± 209	241 ± 307	61 ± 61	40 ± 41
Sesquiterpenoids							
7-α-H-Silphiperfol-5-ene	**1347**	52 ± 58	48 ± 67	103 ± 133	51 ± 78	30 ± 16	117 ± 209
Presilphiperfol-7-ene	**1355**	2 ± 2	4 ± 6	4 ± 5	3 ± 5	3 ± 4	3 ± 4
7-β-H-Silphiperfol-5-ene	**1367**	14 ± 16	14 ± 21	38 ± 49	10 ± 12	9 ± 5	80 ± 190
Silphiperfol-6-ene	**1371**	8 ± 9	8 ± 12	21 ± 28	6 ± 7	5 ± 3	48 ± 116
Silphiperfol-5,7(14)-diene	**1378**	1 ± 1	1 ± 1	3 ± 5	1 ± 2	1 ± 0.3	7 ± 17
β-Caryophyllene	**1446**	3 ± 1	11 ± 12	12 ± 16	5 ± 8	3 ± 1	19 ± 39
α-Farnesene, (*Z,E*)-	**1493**	91 ± 40	79 ± 71	129 ± 194	71 ± 71	52 ± 50	73 ± 40
α-Farnesene, (*E,E*)-	**1508**	134 ± 107	131 ± 120	68 ± 58	112 ± 89	67 ± 90	108 ± 100

Putatively identified volatile compounds	Arithmetic Index*	Uninfested control	*Athalia rosae*	*Plutella xylostella*	*Brevicoryne brassicae*	*Lipaphis erysimi*	*Delia radicum*
		Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD	Peak area / g FW Mean ± SD
Fatty-acid and/or amino-acid derivatives							
3-Hexen-1-ol, acetate, (*Z*)-	**1004**	385 ± 251	554 ± 235	410 ± 355	363 ± 313	667 ± 494	366 ± 288
2-Ethylacetate	**1147**	107 ± 78	71 ± 35	58 ± 39	88 ± 83	53 ± 29	82 ± 83
2-Methylbutanoic acid methyl ester**		29 ± 12	26 ± 10	28 ± 29	28 ± 18	23 ± 15	37 ± 44
3-Hydroxy-2-butanone**		1 ± 1	1 ± 0.4	10 ± 27	0.4 ± 0.2	1 ± 2	2 ± 2
Tiglic aldehyde**		270 ± 197	230 ± 154	240 ± 240	349 ± 298	195 ± 77	373 ± 526
Nitrogen and/or sulphur containing compounds							
unknown thiocyanate	**871**	158 ± 124	136 ± 102	252 ± 270	244 ± 251	175 ± 72	146 ± 123
Allyl isothiocyanate	**885**	1594 ± 830	1440 ± 826	1973 ± 1396	1612 ± 1204	1571 ± 248	1488 ± 873
Benzyl cyanide	**1147**	1071 ± 1048	503 ± 509	397 ± 625	739 ± 595	364 ± 371	653 ± 687
Benzaldehyde, 2-amino	**1235**	1498 ± 1881	597 ± 407	581 ± 499	974 ± 761	579 ± 522	809 ± 356
Methyl thiocyanate**		4 ± 3	3 ± 2	5 ± 4	5 ± 3	3 ± 2	5 ± 4
unknown nitrile m/z 67**		1180 ± 798	1080 ± 440	1165 ± 1022	1307 ± 946	881 ± 297	1008 ± 430
Unknown compounds							
unkonwn m/z 134.18	**1107**	35 ± 29	25 ± 16	17 ± 14	21 ± 15	17 ± 10	23 ± 19
unknown m/z 108.14	**1138**	149 ± 87	154 ± 139	66 ± 75	127 ± 161	114 ± 94	63 ± 38
unknown m/z 150.17	**1429**	28 ± 20	16 ± 17	9 ± 11	24 ± 23	12 ± 16	19 ± 20

* Calculation of Arithmetic Index (AI) as described by Adams (2001) Identification of Essential Oil Components by Gas Chromatography/Mass Spectrometry. USA: Allured Books.

** We did not calculate the arithmetic index for this compound because the shortest chain linear hydrocarbon we injected was octane

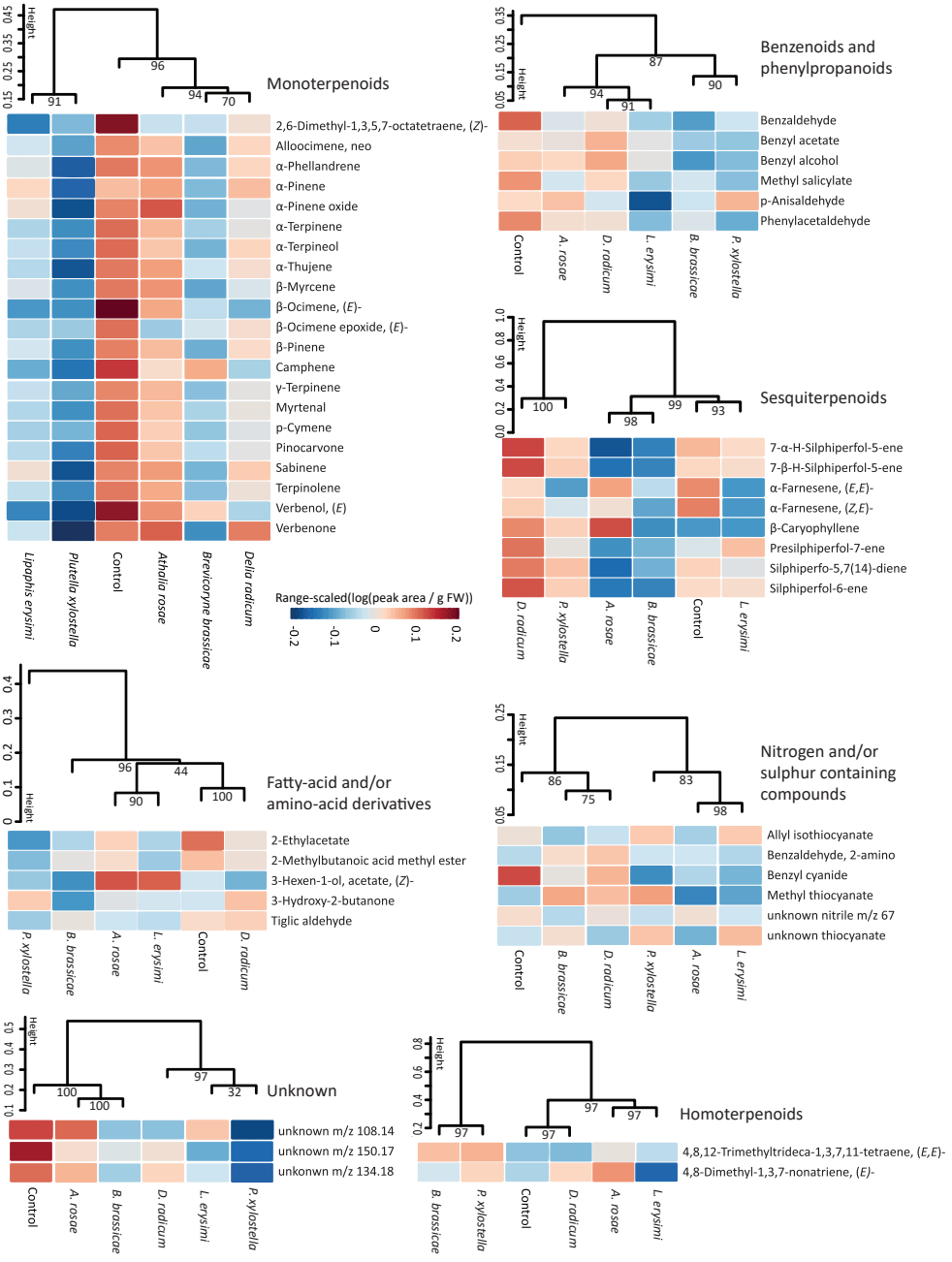


Fig. C7 Dendrogram and heat map of the emission of volatile compounds for each compound class of *Brassica nigra* plants infested with different herbivores or uninfested plants. Dendrogram clustering was performed using Ward's clustering algorithm with Euclidean distances. Values in the dendrogram are approximately unbiased probability values. For the heat map, we used range-scaled log transformed values of volatile emission (peak area / g FW) for each compound. Volatiles were collected after seven days of herbivory. Number of replicates per herbivore treatment varied between seven and nine plants.

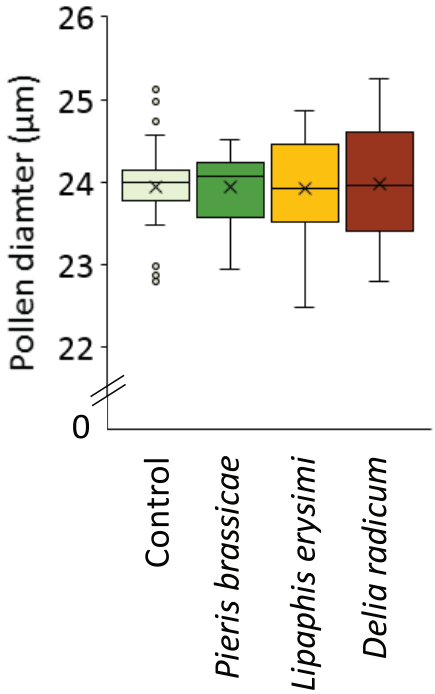


Fig. C8 Size of pollen grains of uninfested *Brassica nigra* plants or plants infested with different herbivores. Boxplots show median (line), mean (x), 1st and 3rd quartiles, minimum and maximum. Outliers (1.5 times the interquartile range below the 1st or above the 3rd quartile) are represented by circles. Measurements were done after seven days of herbivory. Number of replicates per herbivore treatment was 10 plants, per plant we measured pollen for five flowers.

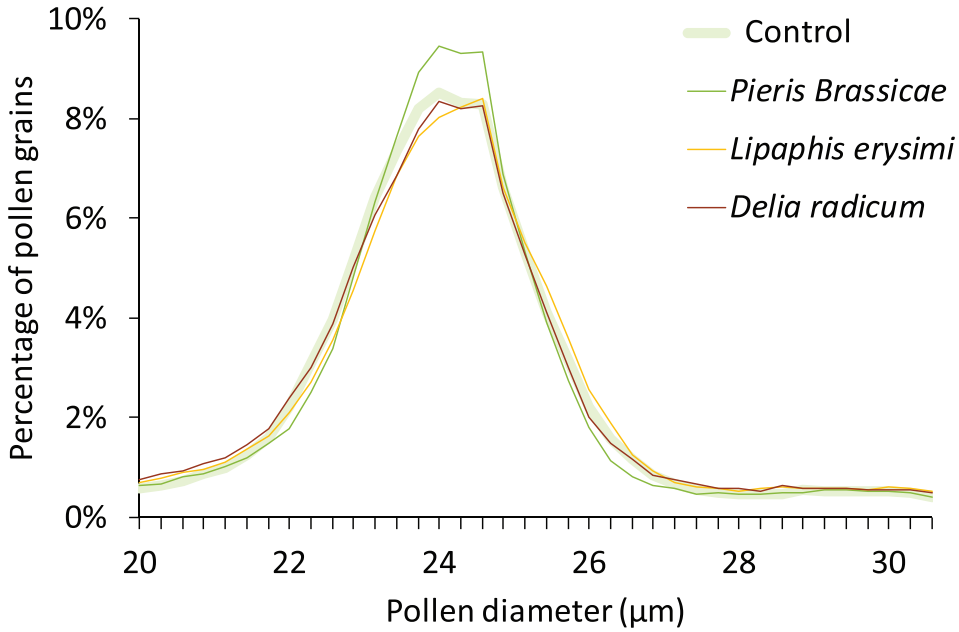


Fig. C9 Size distribution of pollen grains of uninfested *Brassica nigra* plants or plants infested with different herbivores. Measurements were done after seven days of herbivory. Number of replicates per herbivore treatment was 10 plants, per plant we measured pollen for five flowers.

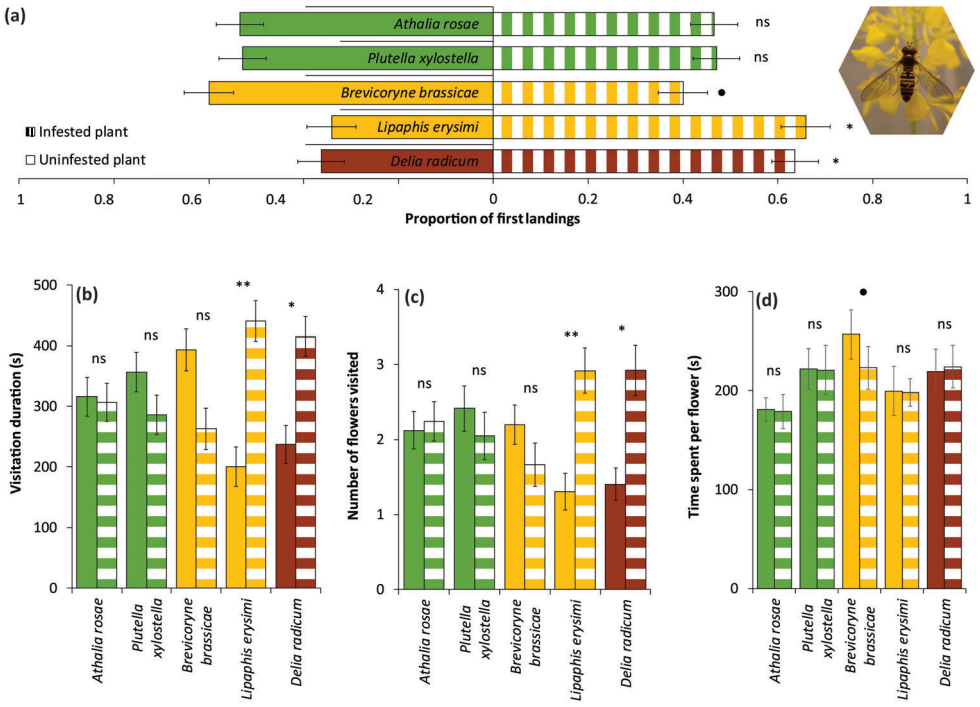


Fig. C10 Preference of the syrphid fly *Episyrphus balteatus* for uninfested *Brassica nigra* plants or plants infested with different herbivores. (a) Proportion of *E. balteatus* syrphid flies (mean ± SE) that first landed on flowers or leaves of *Brassica nigra* plants infested with different herbivores or uninfested plants. b) Visitation duration (mean ± SE); c) number of flowers visited (mean ± SE); and d) time spent per flower (mean ± SE) by individual pollinators on infested or uninfested *B. nigra* plants. Syrphid fly behavior was assessed after seven days of herbivory. Number of replicates per herbivore treatment varied between 85 and 102 syrphid flies, and 9 and 11 plant pairs. Asterisks above bars indicate significant differences with *** = $P < 0.001$, ** = $0.001 \geq P < 0.01$, * = $0.01 \geq P \leq 0.05$, and ● = $0.05 > P < 0.1$, based on Tukey's *post hoc* tests. Photograph shows an *E. balteatus* syrphid fly visiting flowers of *B. nigra*. Photograph credit: Quint Rusman.

Appendix D

Chapter 6
Settling on leaves or flowers: Herbivore feeding site determines the outcome of indirect interactions between herbivores and pollinators

Quint Rusman, Peter N. Karssemeijer, Dani Lucas-Barbosa, and Erik H. Poelman

Submitted

Table D1. Effect of aphid abundance on visitation of the butterfly *Pieris brassicae* and syrphid fly *Episyrphus balteatus* on *Brassica nigra* plants infested with aphids on leaves or flowers. Correlation coefficient (cor) was computed using the Pearson or Kendall method, depending on the distribution of the data. For aphids on leaves + flowers we combined the data for aphids on either leaves or flowers. Bold values indicate results where $P \leq 0.05$. Italic values indicate results where $P \leq 0.1$.

	Brevicoryne brassicae Leaves + flowers			*B. brassicae* Leaves			*B. brassicae* Flowers			*Lipaphis erysimi* Leaves + flowers			*L. erysimi* Leaves			*L. erysimi* Flowers			*Myzus persicae* Leaves + flowers			*M. persicae* Leaves			*M. persicae* Flowers		
	cor	z / t	*P*	cor	z / t	*P*	cor	z / t	*P*	cor	z / t	*P*	cor	z / t	*P*	cor	z / t	*P*	cor	z / t	*P*	cor	z / t	*P*	cor	z / t	*P*
Butterfly visitation																											
Visitation time	0.09	1.20	0.230	0.07	0.46	0.646	-0.02	-0.19	0.850	0.02	0.19	0.846	-0.06	-0.43	0.666	-0.03	-0.21	0.834	0.08	1.07	0.283	-0.11	-0.72	0.475	0.24	1.76	*0.085*
Number of flowers visited	-0.06	-0.57	0.569	-0.10	-0.62	0.536	-0.04	-0.24	0.813	-0.03	-0.34	0.733	0.04	0.27	0.786	-0.12	-0.88	0.385	-0.09	-0.89	0.374	-0.12	-0.81	0.420	-0.10	-0.72	0.476
Time spent per flower	0.09	1.23	0.220	0.16	1.03	0.311	-0.03	-0.24	0.814	-0.04	-0.61	0.545	-0.29	-2.22	**0.031**	0.22	1.56	0.125	0.11	1.60	0.109	-0.13	-0.85	0.398	0.21	1.53	0.133
Syrphid fly visitation																											
Visitation time	0.02	0.24	0.814	0.04	0.29	0.774	0.06	0.55	0.579	-0.10	-1.30	0.195	0.11	0.71	0.479	-0.27	-2.43	**0.015**	0.09	1.25	0.211	0.20	1.37	0.177	0.10	0.92	0.356
Number of flowers visited	0.12	-1.08	0.282	-0.05	-0.30	0.765	-0.19	-1.22	0.228	0.08	0.71	0.480	-0.07	-0.42	0.674	0.05	0.31	0.759	0.13	1.32	0.191	0.06	0.38	0.706	0.23	1.60	0.117
Time spent per flower	0.11	1.31	0.190	-0.05	-0.26	0.800	0.04	0.25	0.807	-0.07	-0.90	0.370	0.01	0.08	0.934	-0.18	-1.18	0.247	-0.04	-0.63	0.532	0.01	0.11	0.914	-0.09	-0.59	0.558

Education statement

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to: Quint Rusman
Date: 12 April 2019
Group: Laboratory of Entomology
University: Wageningen University & Research

1) Start-Up Phase	date	cp
► **First presentation of your project**		
Dealing with herbivores at the expense of pollination: effects of herbivore-induced changes on flower traits.	28 Nov 2014	1.5
► **Writing or rewriting a project proposal**		
Dealing with herbivores at the expense of pollination: effects of herbivore-induced changes on flower traits.	Apr-May 2014	6.0
► **Writing a review or book chapter**		
► **MSc courses**		
Subtotal Start-Up Phase		7.5

2) Scientific Exposure	date	cp
► **EPS PhD student days**		
Get2Gether Annual Experimental Plant Science PhD days, Soest	29-30 Jan 2015	0.6
Get2Gether Annual Experimental Plant Science PhD days, Soest	28-29 Jan 2016	0.6
► **EPS theme symposia**		
Theme 2 Symposium & Willie Commelin Scholten Day, Utrecht University	20 Feb 2015	0.3
Theme 2 Symposium & Willie Commelin Scholten Day, Leiden University	22 Jan 2016	0.3
Theme 2 Symposium & Willie Commelin Scholten Day, Wageningen University	23 Jan 2017	0.3
► **National meetings (e.g. Lunteren days) and other National Platforms**		
Annual Meeting Experimental Plant Sciences, Lunteren	14 Apr 2015	0.3
Annual Meeting Experimental Plant Sciences, Lunteren	09-10 Apr 2018	0.6
Annual Meeting of the Netherlands Entomological Society, Ede	19 Dec 2014	0.3
Annual Meeting of the Netherlands Entomological Society, Ede	18 Dec 2015	0.3
Annual Meeting of the Netherlands Entomological Society, Ede	16 Dec 2016	0.3
Annual Meeting of the Netherlands Entomological Society, Ede	15 Dec 2017	0.3
Netherlands Annual Ecology Meeting, Lunteren	10-11 Feb 2015	0.6
Netherlands Annual Ecology Meeting, Lunteren	09 Feb 2016	0.3
Netherlands Annual Ecology Meeting, Lunteren	13-14 Feb 2018	0.6
► **Seminars (series), workshops and symposia**		
9th Insect-Plant Interaction Workshop, Utrecht	03 Nov 2014	0.3
10th Insect-Plant Interaction Workshop, Amsterdam	19 Nov 2015	0.3
11th Insect-Plant Interaction Workshop, Leiden	22 Nov 2016	0.3
12th Insect-Plant Interaction Workshop, Wageningen	07 Nov 2017	0.3
Current Themes in Ecology, Wageningen	20 Nov 2014	0.3
Current Themes in Ecology, Lunteren	24 Nov 2016	0.3
Current Themes in Ecology, Lunteren	29 Nov 2017	0.3
Student Research Conference, Delft	26 Nov 2014	0.3
KNBV symposium "Het oude continent", Velp	10 Dec 2014	0.3

	Omics Symposium, Wageningen	11 Dec 2014	0.2
	Symposium Van Chemie naar Ecologie, Amsterdam	19 Feb 2016	0.3
	5th Dutch Seed Symposium, Wageningen	04 Oct 2016	0.3
	Plant-Soil-Microbe Interaction Workshop, Wageningen	30 Jun 2016	0.3
	Annual Wageningen PhD Symposium, Wageningen	03 May 2017	0.3
	EPS Symposium Goodbye Ton Bisseling, Wageningen	08 Feb 2017	0.2
	Wageningen Evolution and Ecology Seminars (WEES): Koos Biesmeijer	18 Dec 2014	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Yolanda Chen	21 May 2015	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Jeff Harvey	20 Oct 2016	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Robert Junker	15 Dec 2016	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Mike Singer	12 Apr 2017	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Hans Jacquemyn	01 Jun 2017	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Rich Lenski	31 Aug 2017	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Anne-Nathalie Volkoff	14 Dec 2017	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Jaboury Ghazoul	01 Mar 2018	0.1
	Wageningen Evolution and Ecology Seminars (WEES): Jeff Ollerton	31 May 2018	0.1
	EPS Flying Seminar: Ortrun M. Scheid - Genetics and Epigenetics	19 Nov 2014	0.1
	EPS Flying Seminar: Yves van de Peer: The Evolutionary Significance of Gene and Genome Duplication	03 Feb 2015	0.1
	Seminar Marcelo Carnier Dornelas: Passiflora and Flower Evolution	27 Jan 2015	0.1
	Seminar Florian Schiestl: Ecological Significance of Floral Volatiles	12 Mar 2015	0.1
	Ento-seminar: Joop van Loon, Insect Nutrition	27 Jun 2016	0.1
	Ento-seminar: Eric Schranz, Genetics and Phylogeny	29 Aug 2016	0.1
	Ento-seminar: Michiel Wallis de Vries, Butterfly Conservation	28 Nov 2016	0.1
	Ento-seminar: D.G. Stavenga, Colorful Insect Vision	2016	0.1
	Ento-seminar: Marcel Dicke	24 Apr 2017	0.1
	Plant-Soil Feedback Seminar: Wim van der Putten	13 Nov 2017	0.1
►	**Seminar plus**		
	Wageningen Evolution and Ecology Seminars (WEES) Masterclass: Koos Biesmeijer	18 Dec 2014	0.1
	Wageningen Evolution and Ecology Seminars (WEES) Masterclass: Robert Junker	15 Dec 2016	0.1
	Wageningen Evolution and Ecology Seminars (WEES) Masterclass: Hans Jacquemyn	01 Jun 2017	0.1
	Wageningen Evolution and Ecology Seminars (WEES) Masterclass: Jeff Ollerton	31 May 2018	0.1
	Masterclass Florian Schiestl: Ecological Significance of Floral Volatiles	12 Mar 2015	0.1
►	**International symposia and congresses**		
	15th Symposium on Insect-Plant Interactions, Neuchatel, Switzerland	17-22 Aug 2014	1.5
	16th Symposium on Insect-Plant Interactions, Tours, France	02-06 Jul 2017	1.3
	Gordon Research Conference on Herbivore-Plant Interactions, Ventura, USA	11-17 Feb 2017	2.0
	Ecology and Evolution of Flowers - 2nd International Symposium in Memory of Stefan Vogel, Zürich, Switzerland	24-25 Nov 2018	0.6
►	**Presentations**		
	Student Research Conference (oral)	26 Nov 2014	1.0
	KNBV symposium "Het oude continent" (oral)	10 Dec 2014	1.0
	Netherlands Annual Ecology Meeting (poster)	11 Feb 2015	1.0
	Annual Meeting of the Netherlands Entomological Society (poster)	18 Dec 2015	0.0
	5th Dutch Seed Symposium (oral)	04 Oct 2016	1.0
	11th Insect-Plant Interaction Workshop (oral)	22 Nov 2016	1.0

	Annual Meeting of the Netherlands Entomological Society (oral)	16 Dec 2016	1.0
	16th Symposium on Insect-Plant Interactions (oral)	02-06 Jul 2017	1.0
	Annual Wageningen PhD Symposium (oral)	03 May 2017	1.0
	Gordon Research Conference on Herbivore-Plant Interactions (poster)	15 Feb 2017	1.0
	Netherlands Annual Ecology Meeting (oral)	14 Feb 2018	1.0
	Annual Meeting on Experimental Plant Sciences (oral)	10 Apr 2018	1.0
	Ecology and Evolution of Flowers - 2nd International Symposium in Memory of Stefan Vogel, Zürich (oral)	25 Nov 2018	1.0
►	**IAB interview**		
►	**Excursions**		
	Subtotal Scientific Exposure		29.9

3) In-Depth Studies		*date*	*cp*
►	**EPS courses or other PhD courses**		
	Introduction to R for Statistical Analysis	18-19 May 2015	0.6
	WIAS Advanced Statistics Course Design of Experiments	07-09 Oct 2015	1.0
	European Bee Course, Malta	04-09 Apr 2018	1.5
►	**Journal club**		
	Insect-Plant Interaction discussion group, Laboratory of Entomology	2014-2018	1.0
	Flower and Pollinator discussion group, Laboratory of Entomology	2014-2018	1.0
	PhD discussion group, Laboratory of Entomology	2014-2018	1.0
►	**Individual research training**		
	Subtotal In-Depth Studies		6.1

4) Personal Development		*date*	*cp*
►	**Skill training courses**		
	Competence Assessment	26 Jan 2016	0.3
	EPS Introduction Course	11 Feb 2016	0.3
	Scientific Artwork with Photoshop and Illustrator	01-02 Mar 2016	0.6
	Techniques for Writing and Presenting a Scientific Paper	15-18 Mar 2016	1.2
	Reviewing a Scientific Paper	23 Mar 2017	0.1
	Brain Training	11 Apr 2018	0.3
	Adobe InDesign Essential Training	04-05 Jun 2018	0.6
	The Last Stretch of the PhD Programme	19 Jun 2018	0.0
►	**Organisation of PhD students day, course or conference**		
	12th Insect-Plant Interaction Workshop Organizing Committee	07 Nov 2017	1.5
	Wageningen Evolution and Ecology Seminars (WEES) Organization Committee	2016-2018	1.5
►	**Membership of Board, Committee or PhD council**		
	Subtotal Personal Development		6.4

TOTAL NUMBER OF CREDIT POINTS*	**49.9**

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

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

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