Tales on insect-*flowering* plant interactions

The ecological significance of plant responses to herbivores and pollinators

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To the memory of my father

Abstract

Plants are under natural selection to maximize fitness and are involved in complex interactions with insects. Flowering plants interact with mutualists such as insect pollinators that mediate reproduction and antagonists, such as herbivorous insects. Thus, in nature, plants must balance the investments in defences against their attackers with investments in growth/reproduction, and these investments have been expected to trade-off. A conflict between defence and reproduction can be expected when: 1) primary resources that are allocated to reproduction cannot be allocated to defence and *vice versa*; 2) the attraction of carnivorous insects – natural enemies of the herbivores – conflicts with attraction of pollinators that mediate reproduction. However, despite this potential trade-off, plant reproduction and plant defence have typically been investigated in isolation. In fact, induced defences of flowering plants cannot be fully understood when disconnected from plant-pollinator interactions because selection on plant defensive traits against herbivores implies a plant fitness benefit. This thesis explores the fitness consequences of plant responses induced by herbivores, in the context of plant-mediated interactions with carnivorous and pollinating insects. During this research, field and greenhouse experiments were combined; experiments involving the study of insect behaviour, plant life-history traits and plant chemistry were used to unravel the underlying mechanisms of plant responses to herbivores, while assessing consequences for plant fitness. The study used the Black Mustard, *Brassica nigra,* and the gregarious Large Cabbage White butterfly, *Pieris brassicae,* as the main model organisms. *Brassica nigra* is mainly attacked by specialist herbivores such as *P. brassicae*. *Pieris brassicae* caterpillars initially feed on leaves, where eggs are deposited by the mother butterfly, but the second instar larvae move to the flowers, consuming them entirely, and in large numbers. Results presented in this thesis show that upon exposure to the specialist herbivore, Black Mustard plants reallocate resources and change the profile of defensive compounds in flower tissues. Remarkably, mustard plants sped up reproduction in response to butterfly egg deposition, and compensated for damage caused by the herbivores, while maintaining interactions with pollinators and carnivores. This accelerated seed production benefits the plants because, after hatching, caterpillars quickly move to the inflorescence, and consume flowers, but not the seeds. Reproductive output of these plants, however, was only maintained when interactions with carnivores were preserved. Carnivorous insects can kill up to 95% of *P. brassicae* caterpillars. Not only specialist parasitoids attacked caterpillars, but also generalist predators kill a large proportion of larvae. Here, the results indicate that the interaction with natural enemies of the herbivores is vital in maintaining herbivore pressure low on this wild plant species. In fact, in the absence of carnivores, plants suffered from herbivory in terms of fitness. The data presented in this thesis support the importance of carnivores as a component of a plant's defence strategy. This study reveals some of the underlying mechanisms that allow this short-lived brassicaceous plant to balance investments in defence and reproduction, including mechanisms of plant resistance and re-allocation of resources by plants.

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Chapter 1

Introduction & thesis outline

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Plants are involved in complex interactions with insect herbivores, carnivores and pollinators. Flowering plants have evolved strategies to attract pollinators that are essential for reproduction as well as defensive mechanisms against herbivorous insects (Raguso 2008, Dicke & Baldwin 2010). When regarding reproduction, most flowering plants rely on insects as courier of pollen (Harder & Barrett 2006). Defence against herbivores includes traits that directly affect the plant attacker, but can also be mediated by the attraction of carnivorous insects (Price *et al.* 1980, Schoonhoven *et al.* 2005, Dicke & Baldwin 2010). To maximize fitness, plants are challenged to balance the investments in defences against herbivores with investments in reproduction, and these investments have been hypothesised to trade-off (Herms & Mattson 1992). A trade-off between plant defence and reproduction is expected when: 1) resources that are allocated to reproduction cannot be allocated to defence and *vice versa*; 2) the attraction of carnivorous insects – so-called indirect defence – can conflict with attraction of pollinators that mediate reproduction. However, despite this potential trade-off, plant reproduction and plant defence have typically been investigated in isolation. Herbivore-induced defences by plants in the vegetative stage have been intensively studied (Karban & Baldwin 1997, Dicke & Baldwin 2010, Mumm & Dicke 2010, Karban 2011). Few studies have, however, addressed defences by plants in the flowering stage, despite the fact that flowers directly relate to reproduction, and thus to plant fitness (McCall & Irwin 2006, Kessler & Halitschke 2009, Dicke & Baldwin 2010). Furthermore, flowers may render plants even more attractive to herbivores (Frame 2003, McCall & Irwin 2006), and plant responses to herbivory can affect the behaviour of other members of the plant-associated insect community, including that of pollinating insects (Kessler & Halitschke 2009, Dicke & Baldwin 2010). In fact, induced defences by flowering plants cannot be fully understood when disconnected from plant-pollinator interactions because selection on plant defensive traits against herbivores implies a plant fitness benefit.

The **aim of this thesis project** was to investigate how plants in the flowering stage respond to herbivore attack, and what the consequences are for mutualistic interactions of plants with carnivorous and pollinating insects. Interactions among species are reciprocally related, and therefore, in this thesis I address insect-*flowering* plants interactions from both the plant and the insect perspectives. Ultimately, I aimed to address induced plant responses while assessing fitness consequences for the organisms involved.

Study system

PLANT SPECIES

The Black Mustard, *Brassica nigra* L. (Brassicaceae), is an annual plant of the cabbage family (Brassicaceae) and is considered to be an obligately out-crossing species (Conner & Neumeier 1995). Flowers of *B. nigra* plants have four yellow petals and are hermaphrodite, *i.e.* flowers are composed of both male and female structures. Plants of *B. nigra* produce

hundreds of flowers, and new flowers open daily. Each flower remains open for about 5 days. In nature, flowers of *B. nigra* are pollinated by various insects including bees, syrphid flies and butterflies (Conner & Neumeier 1995). In The Netherlands, this wild species grows as early successional plant and stands in high densities. Like other brassicaceous plants, *B. nigra* contains phenolics and glucosinolates (Smallegange *et al.* 2007, Hussein *et al.* 2010); these are defensive secondary metabolites that protect plants from generalist herbivores (Harborne & Grayer 1993, Schoonhoven *et al.* 2005, Hopkins *et al.* 2009). *Brassica nigra* is, therefore, mainly attacked by specialist herbivores, and some of those prefer to feed on the flowers (Smallegange *et al.* 2007, Bandeili & Müller 2010, Agerbirk *et al.* 2011), which implies direct damage to reproductive tissues. This plant species is a host plant of *Pieris brassicae* L. (Lepidoptera: Pieridae), a commonly observed herbivore on wild and cultivated brassicaceous plants.

SPECIALIST HERBIVOROUS INSECT

The Large Cabbage White Butterfly *P. brassicae* is a specialist herbivore, and adult butterflies search for brassicaceous species to lay eggs. In The Netherlands, there are three generations of *P. brassicae*: the first generation of adult butterflies mainly encounters *B. nigra* plants in their vegetative stage (personal observation). The second and third generations will be exposed to flowering *B. nigra*. *Pieris brassicae* is a gregarious species and female butterflies lay batches of up to 100 eggs on the underside of leaves. When neonate caterpillars hatch, they will initially feed gregariously on leaves of a flowering *B. nigra* plant, but second-instar larvae soon move to flowers (Smallegange *et al.* 2007). Late-instar larvae no longer feed gregariously, but disperse through the inflorescences of *B. nigra* plants, and in this way colonise neighbouring plants as well (Chapters 3 and 6). *Pieris brassicae* caterpillars are voracious feeders and consume flowers of *B. nigra* plants in large numbers. This lepidopteran can cope with the main defensive compounds that brassicaceous plants produce; *Pieris* caterpillars can detoxify glucosinolates and sequester phenolic compounds from brassicaceous plants (Wittstock *et al.* 2004, Ferreres *et al.* 2009, Winde & Wittstock 2011). The fifth instar larvae of *P. brassicae* usually leave the host plant to find a secluded pupation site. The adult feeds on floral nectar of many plant species, including that offered by *B. nigra* flowers. *Pieris brassicae* caterpillars are frequently attacked by various carnivorous insects: 1st and 2nd instar larvae can be attacked by the gregarious parasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae), young larvae can also be preyed upon by ladybird beetles, and later instars can be intensively preyed upon by social wasps such as *Polistes dominula* Christ (Hymenoptera: Vespidae), *Vespula germanica* Fabr. (Hymenoptera: Vespidae), and *V. vulgaris* L. (Hymenoptera: Vespidae) (Chapters 3 and 6).

CARNIVOROUS INSECTS

Cotesia glomerata is a gregarious endoparasitoid that lays its eggs in the caterpillars, where the *C. glomerata* larvae develop. This wasp is a koinobiont parasitoid and, thus, host caterpillars will continue to feed and develop until the final instar is reached (Godfray 1994). Subsequently, wasp larvae emerge from the caterpillars. *Cotesia* larvae spin their cocoons and are provided with an extra layer of silk by the host caterpillar. Cocoons remain attached to the host plant from which a new generation of adult wasps emerges (Geervliet & Brodeur 1992, Mattiacci & Dicke 1995). *Cotesia glomerata* feed on sugar sources during the adult stage. This braconid wasp can parasitize a range of *Pieris* butterfly species, but *P. brassicae* caterpillars are their main host (Geervliet & Brodeur 1992). *Cotesia glomerata* can successfully complete development into adult wasps when parasitizing first or second instar larvae of *P. brassicae* (Geervliet & Brodeur 1992).

Social wasps forage for water, plant fibres, carbohydrates and animal protein. These wasps are generalist predators and forage for animal protein to feed their progeny (Richter 2000). Caterpillars are the main prey for social wasps, although they can also attack other insect larvae, and pollinating insects such as adults of syrphid flies (Chapters 3 and 6). Social wasps can attack prey that are larger than themselves, and in these cases wasps cut the prey in parts that are made into a ball shape before being carried back to the nest (Chapter 6). The number of individuals composing a colony varies significantly between social wasp species: *Polistes* nests are usually composed of fewer than 200 individuals whereas *Vespula* nests can harbour up to a 1000 individuals. A large colony of *Vespula* wasps may consume over 1000 caterpillars per day, and in forests, a nest of *V. vulgaris* may prey upon 0.3 million insects per season (Richter 2000, Picanco *et al.* 2011, Picanco *et al.* 2012).

POLLINATORS

In this thesis project, the behaviour of insect pollinators was observed under greenhouse and field conditions. Bumblebees, honeybees and syrphid flies were the most abundant pollinators observed in the field (Chapters 3 and 7). Greenhouse studies addressed the behaviour of the syphid fly *Episyrphus balteatus* (De Geer) (Diptera: Syrphidae), the honeybee *Apis mellifera* L. (Hymenoptera: Apidae), and the Cabbage White butterflies *P. brassicae* and *P. rapae L.* (Lepidoptera: Pieridae). Like other species of the genus *Apis*, individuals of *A. mellifera* are generalist flower visitors, and have been extensively commercialised for pollination of crops. Honeybees collect both pollen and nectar of *B. nigra* flowers. *Episyrphus balteatus* is one of the most common syrphid fly species worldwide (Jauker & Wolters 2008). Larvae of the syrphid flies feed on aphids, and therefore syrphid flies have been widely used as biological control agents. In the adult stage, *E. balteatus* serve as pollinators; increasing fitness of brassicaceous crop species (Jauker & Wolters 2008). Adult syrphid flies feed on nectar and pollen, but collect mainly pollen from *B. nigra* flowers. In contrast, *Pieris* butterflies feed exclusively on nectar of *B. nigra* flowers.

Outline of this thesis

Chapter 2 reviews the literature on herbivore-induced responses by plants addressing the consequences for insect-flower interactions, *i.e.* in the context of the trade-off between defence and reproduction. More specifically, this chapter addresses how herbivore-induced volatiles emitted by plants in the flowering stage can influence the plant-associated insect community, including the effects of herbivory on host plant selection by adult herbivores, host location behaviour of parasitoids, and foraging preferences of pollinators.

Chapter 3 addresses the effects of herbivore infestation by the specialist *P. brassicae* on pollinator behaviour and seed production. Responses of *B. nigra* plants to herbivore exposure were investigated since egg deposition, throughout larval development. In this field study, herbivores were exposed to naturally occurring predators and parasitoids, and dispersal and mortality of caterpillars during the experiments were recorded. The main goal was to evaluate the overall effects of herbivory on plant fitness.

Chapters 4 and 5 question whether and how induced plant responses to herbivory can lead to changes in pollinator behaviour. Chapter 4 focuses on the systemic effects of herbivore damage to leaves on floral reward and flower traits exploited by pollinators, considering the role of defensive chemistry against herbivores on pollinator behaviour. Pollinator visitation is influenced by the quality and quantity of pollinator rewards, such as floral nectar, and by flower traits that can be associated with the value of such rewards. In this chapter, the systemic effects of responses to insect herbivory on flower volatile emission and nectar production were investigated; the aim was to infer whether changes in odours exploited as cues by pollinators, could be associated with actual changes in quantity and quality of nectar offered by *B. nigra* flowers.

Chapter 5 further examines the mechanisms through which herbivore-induced responses by plants can affect pollinator behaviour. This chapter addresses not only induced phytochemical responses to herbivory, but also plant responses to pollination. Plants can respond to the activities of pollinators and herbivores, and the same classes of plant secondary metabolites associated with induced responses to herbivory can also be associated with pollinator attraction. This chapter addresses how *B. nigra* plants respond to pollination and insect herbivory in terms of odours and flower pigments, and whether plant responses to herbivory interfere with responses to pollination. I considered responses of plants to folivorous and florivorous larvae of *P. brassicae.*

In Chapter 6, I take the herbivore perspective to investigate why herbivores feed on the flowers. This chapter explores whether flowers can provide caterpillars with a refuge from their natural enemies and what the consequences are for the survival of *P. brassicae* when feeding on flowering plants. Field and greenhouse experiments were used to investigate how flowers influence host-plant selection by adult herbivores, and subsequent interactions of the herbivore with parasitoids and predators on different parts of the flowering plant. Results are discussed in the context of theories of diet breadth and enemy-free space, and in terms of the selective pressures and constraints on herbivore survival in nature.

Chapter 7 addresses how *B. nigra* plants balance investments between reproduction and defensive strategies. This chapter explores both the allocation of resources by the plants upon herbivory and the role of carnivorous insects as a defence strategy.

In Chapter 8, the findings of this thesis are integrated. The ecological significance of induced responses to herbivory and pollination are discussed, and I emphasise that to fully understand how plant defence strategies evolved, it is necessary to integrate studies on plant-herbivore and plant-pollinator interactions. Ultimately, such studies need to be taken all the way to the level of plant fitness. The findings of this thesis, together with recently published studies by others, provide the first steps in this direction.

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Chapter 2

The effects of herbivore-induced plant volatiles on flower visiting insects - a review

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Abstract

Plants are faced with a trade-off between on the one hand growth, development and reproduction and on the other hand defence against environmental stresses. Yet, research on insect–plant interactions has addressed plant–pollinator interactions and plant–attacker interactions separately. Plants have evolved a high diversity of constitutive and induced responses to attack, including the systemic emission of herbivore-induced plant volatiles (HIPVs). The effect of HIPVs on the behaviour of carnivorous insects has received ample attention for leaf-feeding (folivorous) species and their parasitoids and predators. Here, we review whether and to what extent HIPVs affect the interaction of plants in the flowering stage with mutualistic and antagonistic insects. Whereas the role of flower volatiles in the interactions between plants and insect pollinators has received increased attention over the last decade, studies addressing both HIPVs and pollinator behaviour are rare, despite the fact that in a number of plant species herbivory is known to affect flower traits, including size, nectar secretion and composition. In addition, folivory and florivory can also result in significant changes in flower volatile emission and in most systems investigated, pollinator visitation decreased, although exceptions have been found. Negative effects of HIPVs on pollinator visitation rates likely exert negative selection pressure on HIPV emission. The systemic nature of herbivore-induced plant responses and the behavioural responses of antagonistic and mutualistic insects requires the study of volatile emission of entire plants in the flowering stage. We conclude that approaches to integrate the study of plant defences and pollination are essential to advance plant biology, in particular in the context of the trade-off between defence and growth/reproduction.

Keywords: glucosinolates, green-leaf volatiles, herbivores, indirect defence, induced defence, parasitoids, pollinators, terpenoids.

Trade-off between growth, development and reproduction *versus* defence

Plants have been under natural selection to maximise their fitness, resulting in a dynamic balance between growth and defence that is affected by the environmental conditions experienced by the plant. A central paradigm in plant biology is the existence of a trade-off between on the one hand growth, development and reproduction and on the other hand defence against environmental stresses (Herms & Mattson 1992). Thus, energy invested in defence cannot be invested in growth, development and reproduction and *vice versa*.

Plants are exposed to a multitude of attackers. The number of insect species is estimated to be about 6 million, half of them being herbivorous (Schoonhoven *et al.* 2005). Arthropod–plant interactions are characterised by a high degree of trophic specialism: about 80% of herbivorous arthropods are specialist feeders that accept only a limited number of plant species belonging to a single genus or family. To combat attackers, plants have evolved an astounding diversity of phytochemical defences. More than 100,000 secondary compounds of plant origin have been recorded and there is ample evidence for the role of secondary metabolites in the defence of plants against herbivorous insects. Many plant families produce characteristic secondary metabolites such as glucosinolates in the Brassicaceae (Hopkins *et al.* 2009) or alkaloids in the Solanaceae (Pomilio *et al.* 2008). Phytochemical–based plant defence may be constitutive, causing resistance to the large majority of herbivorous species, except for a small number of specialist feeders. Moreover, herbivory can induce additional direct and indirect defences (Karban & Baldwin 1997, Gardner & Agrawal 2002, Kessler & Baldwin 2002, Dicke & Baldwin 2010) such as, for instance, the production of herbivore-induced plant volatiles (HIPVs) that attract carnivorous enemies of herbivores (Dicke & Baldwin, 2010). It is generally assumed that the benefit of inducible over constitutive defences is cost reduction (Karban & Baldwin 1997), especially in terms of biosynthetic costs. However, plants may also incur ecological costs related to defence investments. For instance, secondary metabolites may be exploited by specialist herbivores during host-plant selection (Hopkins *et al.* 2009), or herbivores may sequester plant secondary metabolites and exploit them for their own defence against carnivorous enemies (Nishida 2002). As a result of such biosynthetic and ecological costs, plants will be under selection to optimise the balance between investments in constitutive and inducible defences. An additional benefit of inducible defences is that the plant has a plastic phenotype which is likely to retard adaptation in herbivores (Agrawal & Karban 1999). However, the costs of induced defences have rarely been considered in the context of the trade-off between reproduction and defence (Agrawal *et al.* 1999). The expression of defences may affect, for instance, a plant's interactions with pollinators and when induced defence reduces pollinator visitation, this represents another ecological cost. In this review we will focus on the consequences of induced indirect defence for insect–flower interactions, i.e. in the context of the defence *vs.* growth/reproduction trade-off. More specifically, we address how HIPVs of plants in the flowering stage may influence (a) host plant selection by adult herbivores, (b) host location by parasitoids, and (c) foraging preferences of pollinators.

Induced direct and indirect defences

Two types of induced plant defence are distinguished: (a) direct defence that affects the performance or behaviour of its attacker directly, *e.g.* through an increased concentration of secondary metabolites (Steppuhn *et al.* 2004, Gols *et al.* 2008), including plant volatiles (De Moraes *et al.* 2001, Kessler & Baldwin 2001) and (b) indirect defence that enhances the effectiveness of natural enemies of herbivores through the production of HIPV (Dicke & Baldwin 2010), and through the induction of extrafloral nectar (EFN) (Heil 2008, Dicke 2009). The induced production of volatile organic compounds (VOCs) that attract carnivorous arthropods can occur in response to herbivore feeding damage (Vet & Dicke 1992) or egg deposition (Hilker & Meiners 2006), both aboveground (Arimura *et al.* 2005) and belowground (Erb *et al.* 2009a). The emission of such HIPVs has been documented for plant responses to insects belonging to five orders (Lepidoptera, Diptera, Thysanoptera, Coleoptera and Hemiptera) and mites (Mumm & Dicke 2010). HIPVs include compounds produced through various biosynthetic pathways, including the octadecanoid pathway leading to fatty-acid derived green leaf volatiles (GLVs), the MEP (methyl-erythritol phosphate) pathway leading to monoterpenes and diterpenes, the mevalonate pathway giving rise to sesquiterpenes, and the shikimate pathway leading to a large diversity of aromatic metabolites (Fig. 1). Moreover, secondary metabolites may be enzymatically converted to volatile compounds such as isothiocyanates (Fig. 1) and nitriles derived from glucosinolates in the Brassicaceae or cyanides derived from cyanogenic glucosides in the Papilionaceae (Schoonhoven *et al.* 2005). HIPVs constitute complex mixtures of up to 200 compounds and our knowledge on the active components of such mixtures is still limited (D'Alessandro *et al.* 2006). The activity of individual compounds may depend on the context of the total blend (De Boer *et al.* 2004, Schröder & Hilker 2008). The emission of HIPVs is a systemic

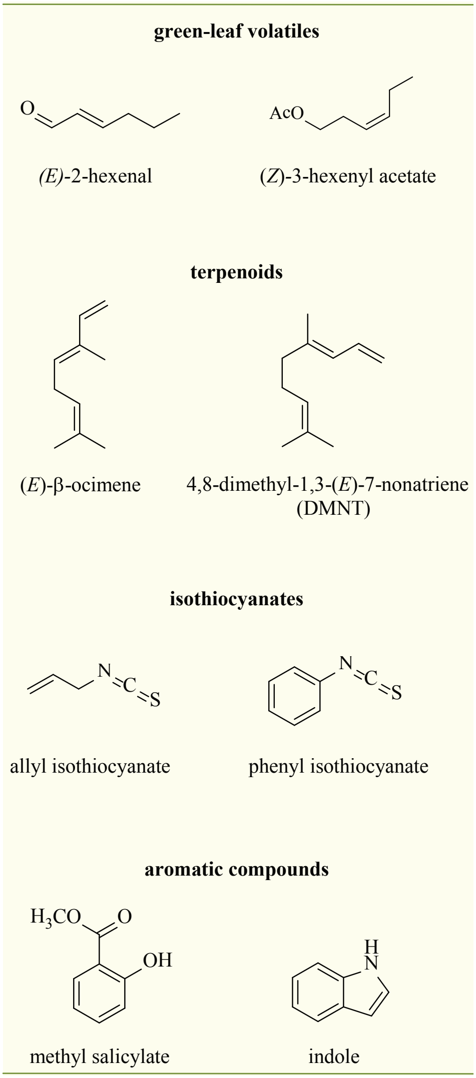

Fig. 1 Examples of HIPVs produced through different biosynthetic pathways or that are catalytic products of plant secondary metabolites.

phenomenon and this has been especially studied for aboveground tissues (Heil & Ton 2008), but may also connect above- and belowground tissues (Dicke & Dijkman 2001, Erb *et al.* 2009a). The induction of extrafloral nectar production that leads to enhanced visitation by carnivores such as ants and parasitoids (Heil 2008), can also be systemic (Wäckers & Wunderlin 1999).

In sum, induced indirect defences may enhance the abundance and activity of carnivorous arthropods and, consequently, make a plant a carnivore-dense space.

Herbivore-induced plant volatiles in a community context

Plants are members of complex communities consisting of mutualists and antagonists at different trophic levels (Kessler & Baldwin 2007, Pieterse & Dicke 2007, Poelman *et al.* 2008). Many of these community members interact with the plant, *e.g.* through trophic interactions, through infochemically-mediated interactions or through other trait-mediated indirect interactions (Ohgushi *et al.* 2007, Poelman *et al.* 2008). A change in a plant's phenotype consequently affects a range of interactions within the food web and, therefore, each trait change is subjected to a complex set of selection pressures. For instance, when a plant initiates the emission of HIPVs, any organism in the environment may respond to this chemical information. Indeed, responses are known from herbivores, carnivores, plant pathogens, and neighbouring plants (Baldwin *et al.* 2006, Erb *et al.* 2009b, Dicke & Baldwin 2010), and may result in attraction or repellence of multiple plant community members. A trade-off might arise from infochemically mediated interactions, because both mutualistic and antagonistic species might exploit the same phytochemical responses (Dicke & Baldwin 2010). Most research on HIPVs has addressed the responses of carnivorous arthropods (Mumm & Dicke 2010) while responses of other community members have begun to attract more interest (Dicke & Baldwin 2010, Kessler & Heil 2011).

Herbivore-induced changes in plants in the flowering stage

Most of the research on herbivory-induced responses of plants has addressed vegetative plants. Herbivore-induced changes in plants in the flowering stage have received much less attention and only recently have an increasing number of studies addressed the effect of herbivory on traits of flowering plants. Florivory and folivory may both have consequences for plant fitness. Florivory can significantly affect plant reproductive success by reducing the number of reproductive structures available or by affecting their function (McCall & Irwin 2006, Strauss & Whittall 2006, Wise & Cummins 2007, Zangerl & Berenbaum 2009, Cardel & Koptur 2010). Feeding by four caterpillars of *Pieris brassicae* on the flowers of a *Brassica nigra* plant reduced seed production by 50–80% (Smallegange *et al.* 2008), and a single late-instar parsnip webworm *Depressaria pastinacella* feeding on wild parsnip reduced the number of female flowers and seed set by roughly 50% (Zangerl & Berenbaum 2009).

Plant reproductive success may even be affected when only a small part of the flower is consumed, as was reported for *Isomeris arborea* flowers fed upon by the pollen beetle *Meligethes rufimanus* (Krupnick & Weis 1999).

Moreover, damage by folivores can also lead to changes in flower morphology and metabolism (Quesada *et al.* 1995, Strauss & Armbruster 1997, Mothershead & Marquis 2000, Adler *et al.* 2001, Poveda *et al.* 2003, 2005). Folivory can reduce floral rewards, such as nectar and pollen that are exploited by flower visitors (Strauss 1997, Bruinsma *et al.* 2008, Samocha & Sternberg 2010). Leaf herbivory can result in smaller flowers and fewer open flowers (Mothershead & Marquis 2000, Adler *et al.* 2001). Herbivory in an early stage of plant development may result in smaller plants with a delayed and shorter flowering period (Poveda *et al.* 2003), and impacts on seed and fruit set might differ depending on infestation time (Oguro & Sakai 2009). This is a good illustration of the trade-off between defence and reproduction that a plant faces.

Furthermore, folivory or florivory may also alter flower chemistry. Plants may respond to herbivory by increasing toxin concentrations in nectar and flower tissues (Euler & Baldwin 1996, Adler *et al.* 2006, McCall 2006), and by producing HIPVs in the flowers (Röse & Tumlinson 2004, Kessler & Halitschke 2009, Zangerl & Berenbaum 2009). Just as has been reported for plants in the vegetative stage, phytochemical responses of plants in the flowering stage to herbivory can have a systemic nature (Heil & Ton 2008). For example, mechanically damaged leaves increased the emission of volatiles by *Cucurbita pepo* subsp. *texana* flowers (Theis *et al.* 2009). Only a few studies have addressed the induced emission of floral volatiles after insect folivory (Effmert *et al.* 2008, Kessler & Halitschke 2009) leading to different conclusions as we discuss in the next section. Even when flower volatile emission is not affected (Effmert *et al.* 2008), HIPVs locally produced by leaves may alter the blend of the entire plant and influence the behaviour of flower visitors. Systemic effects may also operate in response to herbivory to the reproductive organs. For instance, herbivory by the lepidopteran *Helicoverpa zea* on cotton flower buds increased the emission of volatiles from leaves of the same plant (Röse & Tumlinson 2004). The systemic volatile blend emitted by leaves differed significantly, however, from the volatile blend locally emitted by the damaged cotton buds. Currently studies on systemic effects of herbivory on HIPV emission have mostly focussed on vegetative plants. Extending this to plants in the flowering stage will be important to investigate the interaction between plant defence and pollination.

Chemistry of herbivore-induced plant volatiles – flowers *versus* leaves

Flower volatiles, classified according to their chemical structure, are found in seven major classes of compounds: aliphatics, benzenoids/phenyl propanoids, C5-branched compounds, terpenoids, nitrogen-containing compounds, sulphur-containing compounds, and a class of various cyclic compounds (Knudsen *et al.* 2006). Most foliage-emitted HIPVs fall into the same seven categories (Mumm & Dicke 2010) (Fig. 1). Phytochemical responses to herbivory have been well studied, *e.g.* in brassicaceous and solanaceous plants. We selected a number of plant species of both plant families to provide insight in what is known about HIPVs when compared to constitutive leaf and flower compounds (See supporting information in Appendix A, Tables A1a and b). It is important to notice that at the level of major compound classes, constituents of all the seven classes can be constitutively found in the volatile blends of brassicaceous and solanaceous plants (Knudsen *et al.* 2006). This suggests that plant volatile secondary metabolite biosynthesis, including that giving rise to floral scents, is not phylogenetically constrained at the family level (Knudsen *et al.* 2006). At the level of individual metabolites, however, volatile composition differs significantly among closely related species (Tables A1a and b) (Tollsten & Bergström 1988, Jürgens *et al.* 2002, Raguso *et al.* 2003).

Plants in the vegetative stage may emit HIPVs that are also emitted during the flowering stage. The homoterpene 4,8-dimethyl-1,3,7-nonatriene (DMNT), clearly involved in the attraction of predators and parasitoids (Dicke *et al.* 1990, Turlings *et al.* 1990, Dicke 1994, Kappers *et al.* 2005, Mumm & Dicke 2010), was induced by folivory in brassicaceous plants (Soler *et al.* 2007, Abel *et al.* 2009) and in the solanaceous *Solanum lycopersicum,* a cultivated tomato species (Kant *et al.* 2004). Moreover, this homoterpene is emitted by various flowers (Kaiser 1993), and may be exploited by both pollinators and adult herbivores (Svensson *et al.* 2005, Wiemer *et al.* 2009). The benzenoid methyl salicylate is synthesised *de novo* by leaves of *Nicotiana attenuata* plants that have been exposed to herbivory (Kessler & Baldwin 2001) and is also constitutively emitted by flowers of another solanaceous species, *i.e Solanum peruvianum* (Kessler & Halitschke 2009).

The production of HIPV has been extensively demonstrated for vegetative plants, but our knowledge on herbivore-induced flower volatiles (HIFV) is still limited. Although the Solanaceae and Brassicaceae are among the best studied plant families with regard to induced plant response (Tables A1a and b), we found only two studies that have investigated whether the emission of flower volatiles is altered after herbivory (Tables A1b) (Effmert *et al.* 2008, Kessler & Halitschke 2009). *Manduca sexta* damage did not significantly increase floral volatile production by *Nicotiana suaveolens* (Effmert *et al.* 2008). In contrast, the floral volatile blend of wild tomato plants (*S. peruvianum*) whose leaves were damaged by this herbivore differed significantly from that of non-damaged plants (Kessler & Halitschke 2009). For other plant families we found only three studies that investigated whether leaf or flower herbivory can induce the biosynthesis of flower VOCs (Röse & Tumlinson 2004, Theis *et al.* 2009, Zangerl & Berenbaum 2009). These studies have demonstrated that the induction might be systemic and flowers may also produce VOCs *de novo* in response to herbivory (Appendix A, Table A1c). Most HIPVs produced by damaged cotton leaves in vegetative plants were also emitted at higher rates by cotton flower buds (Table A1c) (Loughrin *et al.* 1994, Röse & Tumlinson 2004).

Folivory may not only induce the emission of floral volatiles, it may also reduce floral scent emission. Folivory by *M. sexta* larvae on *N. attenuata* plants resulted in flowers with a reduced emission of the floral volatile benzyl acetone, a compound that attracts night-active hawkmoths such as *M. sexta* (Kessler et al. 2010). This effect is elicited by oral secretions of the caterpillars and requires jasmonic acid (JA)-mediated signal transduction. Interestingly, JA also mediates the induction of defences such as nicotine production and HIPV production in *N. attenuata* (Kessler & Baldwin, 2002).

Flower volatiles and the systemic emission of HIPVs may affect different members of the insect community. In the next three sections, we will discuss how changes in volatile emission due to flowering and herbivory may affect insects at different trophic levels.

Interactions between herbivores and plants in the flowering stage

The adults of many species of insect herbivores forage on flowers, including the flowers of their larval host plants. Nutritious rewards that flowers offer and their advertisements, including plant volatiles, can influence herbivore foraging behaviour (Wäckers *et al.* 2007). Lepidopterans that are attracted to flower volatiles might not only feed on nectar, but may also lay their eggs on the same plants (Reisenman *et al.* 2010). The larvae that hatch from these eggs may not restrict feeding to the leaves but may also move to the inflorescence and feed on the flowers. For instance, *Pieris brassicae* caterpillars migrate from leaves to flowers late in the second instar and become florivorous (Fig. 2), even though the flowers contain fivefold higher levels of glucosinolates than leaves (Smallegange *et al.* 2007). Higher levels of glucosinolates in the flowers may protect *P. brassicae* caterpillars from generalist predators (Rayor *et al.* 2007). Larvae of the another pierid butterfly, *Anthocharis cardamines,* also a Brassicaceae specialist, preferentially feed on flowers, as a consequence of their mother's oviposition preference for floral parts (Agerbirk *et al.* 2011).

Fig. 2 Fifth instar larvae of *Pieris brassicae* feeding on a leaf (a) and on flower buds (b) of *Brassica nigra* plants. (courtesy Nina E. Fatouros).

Oviposition preferences of adult herbivores may differ depending on whether the plant is in the vegetative or flowering stage. Floral scent may render plants more attractive to adult herbivores. The same herbivore species that oviposits on a plant and thus exposes the plants to herbivorous larvae may act as plant pollinator (Adler & Bronstein 2004, Bronstein *et al.* 2009). This antagonistic–mutualistic association may be infochemically mediated. In the field, higher numbers of *M. sexta* eggs were consistently recorded on *Datura wrightii* plants that had flowers (Reisenman *et al.* 2010). The presence of (+)-linalool in the volatile blend of the flowers enhanced oviposition by the moths. However, when (–)-linalool was present, the mixtures mimicking flower scent attracted female moths for feeding, but not for oviposition (Reisenman *et al.* 2010). These studies illustrate that the addition of a new compound or a higher concentration of a single component of a complex floral odour mixture, may change insect behavioural responses and thereby the insect–plant interaction.

Also herbivory can lead to changes in host-plant selection by adult herbivores. However, this has so far mainly been investigated for vegetative plants (De Moraes *et al.* 2001, Kessler & Baldwin 2001, Schoonhoven *et al.* 2005).

Effects of herbivory on pollinator-flower interactions

Of all flowering plant species about 66% depend on insect pollination for their reproduction (Myers 1996, Schoonhoven *et al.* 2005, Klein *et al.* 2007). When herbivory results in modification of flower traits, pollinator visitation can be expected to change as well. Plant investments in herbivore-induced defence may negatively affect investments in both vegetative and reproductive tissues, including the production of pollen and nectar as pollinator rewards. In recent years, it has been demonstrated that herbivory can lead to divergent consequences for pollinator attraction. For example, root herbivory increased visits by pollinators to flowers of the mustard *Sinapis arvensis* (Poveda *et al.* 2003, 2005). Combined leaf and root herbivory reduced flowering period and number of fruits produced, but seed production was not affected (Poveda *et al.* 2003). In most studies, however, folivory and florivory decreased pollinator visitation (Strauss *et al.* 1996, Lehtilä & Strauss 1997, Krupnick & Weis 1999, Adler *et al.* 2001, Hamback 2001, Kessler & Halitschke 2009, Cardel & Koptur 2010, Danderson & Molano-Flores 2010). Bees and syrphid flies visited non-damaged wild radish plants more frequently than herbivore-infested plants (Lehtilä & Strauss 1997). The lower preference observed for bees was ascribed to fewer and smaller flowers. However, syrphid fly preference could not be correlated to changes in the morphological flower traits measured. These findings suggest that the syrphid flies may have used chemical cues to discriminate between damaged and non-damaged plants.

Plant odours influence the foraging behaviour of pollinators (Kessler & Halitschke 2007, Kessler & Baldwin 2007, Raguso 2008), and the role of HIPVs in the observed changes in pollinator behaviour need to be further investigated (Kessler & Halitschke 2007, Dicke & Baldwin 2010). Lately, attention has been paid to understand to what extent changes in olfactory cues emitted by herbivore-damaged flowering plants can influence pollinator behaviour. For *Cucurbita pepo* subsp. *texana* plants no effects of flower number, display or quality of the rewards on pollinator behaviour were recorded. Pollinator visitation, however, was reduced in response to changes in the volatile emission rate of flowers after the leaves had been mechanically damaged (Theis *et al.* 2009). Pollinators made fewer visits to and spent less time on wild tomato plants whose leaves were damaged by an insect herbivore (Kessler & Halitschke 2009). Floral volatile emission by wild tomato plants that were damaged on the leaves by *Manduca sexta* caterpillars differed significantly from that produced by non-damaged plants (Table A1b). These results indicate that local and systemic HIPV emission may influence pollinator foraging behaviour, and when pollinator visitation is negatively affected, this exerts a negative selection pressure on HIPV emission (Dicke & Baldwin 2010). Reduced emission rates of flower volatiles may supposedly render plants less attractive to pollinating insects. On the other hand, for an individual flowering plant under attack, it would be beneficial to attract pollinators and ensure reproduction. Loss of resources due to herbivory could be compensated for by enhancing interactions with mutualists (Strauss & Murch 2004), and such mechanisms can be expected to evolve in obligately outcrossing species (Abel *et al.* 2009).

An exciting study on the effects of folivory on the interactions of flowering plants with pollinators shows how plants may deal with pollinators that pose a risk of being a herbivore as well: folivory on *N. attenuata* plants by hawkmoth larvae results in a shift of pollinator species as a result of a change in time of flower opening and a change in flower volatile emission (Kessler *et al.* 2010). As a consequence, the flowers are now pollinated in the morning by hummingbirds instead of by hawkmoths that not only feed on nectar but also deposit their eggs on the plants (Kessler *et al.*, 2010). This exciting study clearly connects to the trade-off between reproduction and defence.

Effects of herbivory on parasitoid-flower interactions

Flowering plants provide parasitoids with nutritious nectar and, possibly, hosts. Thus, parasitoids may benefit from visiting flowering plants both for feeding and host finding. Adult parasitoids feed on nectar, and their food-searching behaviour depends on their satiety status (Wäckers 1994, Kugimiya *et al.* 2010). For instance, in contrast to satiated *Cotesia vestalis,* starved wasps were attracted to *Brassica rapa* flowers by olfactory and visual cues (Kugimiya *et al.* 2010). However, whether herbivory influences the attraction of parasitoids to the flowers remains to be investigated.

Phradis interstitialis parasitoid wasps were attracted to odours emitted by buds of *B. napus*, in which their hosts, the eggs and first instar larvae of *Meligethes* pollen beetles, reside. The parasitoids use their long and thin ovipositor to penetrate the flower bud walls of *B. napus* and oviposit in eggs or first instar larvae. The parasitoids avoided odours emitted by open flowers. In contrast, another parasitoid wasp of the pollen beetles, *Tersilochus heterocerus,* did not differentially respond to odours emitted by buds and flowers of *B. napus* alone; however, they were more attracted to flower odours contrasted to yellow colour stimuli than to bud odours contrasted to green stimuli (Jonsson *et al.* 2005). With their short and thick ovipositor, *T. heterocerus* is known to prefer to lay eggs in second larval instars of pollen beetles; hosts in this developmental stage are more frequently found in open flowers (Jonsson *et al.* 2005). These data show that parasitoids use flower volatiles when foraging for hosts. It would be interesting to further investigate whether bud and flower scents produced by *B. napus* are altered after herbivore oviposition or feeding damage, and whether such changes influence parasitoid attraction to intact buds or flowers when compared to host-damaged buds or flowers. Only one study so far addressed the role of HIPV emitted by herbivore-damaged flowers on parasitoid foraging behaviour. The parasitoid wasp *Apanteles taragamae* uses HIPVs produced by damaged cowpea flowers to locate the legume pod borer *Maruca vitrata*. The wasps were significantly more attracted to odours produced by cowpea flowers infested with the legume pod borer larvae than to odours of non-infested or mechanically damaged flowers (Dannon *et al.* 2010). This study should stimulate further studies on the role of herbivore-induced flower volatiles in the interactions of plants with members of the insect community as well as the underlying mechanisms such as signal-transduction pathways (see *e.g.* Kessler *et al.*, 2010).

Conclusion and future perspectives

There is ample evidence for induced direct and indirect defences of plants (Kessler & Baldwin 2002, Mumm & Dicke 2010) and how these affect members of the associated insect community such as carnivores, herbivores and neighbouring plants (Kessler & Halitschke 2007, Dicke & Baldwin 2010). The vast majority of these studies have addressed vegetative plants and only very few investigated how the production of HIPVs may affect plant fitness (van Loon *et al.* 2000, Fritzsche-Hoballah & Turlings 2001, Smallegange *et al.* 2008). In addition, there is ample evidence for interactions between plants and pollinators and these, self-evidently, address flowering plants (Kessler & Halitschke 2007, Kessler & Baldwin 2007, Kessler *et al.* 2008, Raguso 2008, 2009, Baldwin 2010). In the context of the important trade-off between defence and growth/reproduction (Herms & Mattson 1992), approaches to integrate the study of plant defences and pollination is an essential next step to advance plant biology (Kessler *et al.* 2008). Flowering plants differ in many physiological and biochemical aspects from vegetative plants and, consequently, induced responses to herbivory may be different from responses to herbivory in vegetative plants.

In this review we have discussed how herbivore-induced defences affect interactions between flowering plants and insects, with an emphasis on pollinators, herbivores and carnivores. There is ample evidence that insect herbivory, including root herbivory, folivory and florivory can change various flower traits, including size, amount of pollen and nectar volume (Strauss *et al.* 1996, Lehtilä & Strauss 1997, Krupnick & Weis 1999, Adler *et al.*

2001, Hamback 2001, Kessler & Halitschke 2009, Cardel & Koptur 2010, Danderson & Molano-Flores 2010). Such changes in flower traits can directly affect pollinator behaviour. Moreover, insect herbivory can also change the emission of plant volatiles from leaves, roots and flowers. The compounds emitted by flowers of insect-damaged plants overlap with the compounds emitted by plant leaves. However, only few studies have addressed the effects of insect herbivory on the emission of plant volatiles from flowers and the resulting effects on pollinators (Effmert *et al.* 2008, Kessler & Halitschke 2009). For vegetative plants, it is well documented that herbivory alters various plant traits, including HIPV emission and extrafloral nectar production and that these phenotypic changes have a major effect on community dynamics (Kessler & Halitschke 2007, Poelman *et al.* 2008, Dicke & Baldwin 2010). Moreover, also the emission of floral scents can influence different members of the associated insect community, such as nectar robbers and florivores (Baldwin 2010). Floral scent can also provide information on pollination status and nectar reward (Kessler & Halitschke, 2007; Kessler *et al.* 2008, 2010).

Due to the systemic nature of herbivore-induced plant responses, studying volatile emission of vegetative and flower tissues separately provides an incomplete picture. Moreover, at a distance, floral scents and foliar scents are likely to mix and community members will be exposed to both. How this affects their behavioural responses remains to be investigated. Recently, studies on induced plant defences have taken an integrative approach towards aboveground and belowground interactions (van der Putten *et al.* 2001, Erb *et al.* 2009b, Pineda *et al.* 2010), towards defences against pathogens and herbivores (Pieterse & Dicke 2007) and towards plant responses to multiple attackers (Dicke *et al.* 2009). Studies that address the interaction between induced defences and pollination are badly needed and indeed an exciting first study on this has recently been published (Kessler & Halitschke 2009). Proceeding in this area will provide major progress towards understanding the ultimate consequences of induced defences, i.e. the effects of herbivory-induced plant responses on plant fitness (see discussion in Dicke & Baldwin, 2010; Kessler & Heil, 2010). By increasing the complexity of the systems studied, the view on the selection pressures on induced phytochemical defences such as induced indirect defence becomes increasingly complex as well. However, by addressing the effects on the different individual community members that exert different selection pressures, as well as the effects on seed production, an important step will be made to address the central paradigm of plant biology, i.e. the trade-off between defence and growth/reproduction (Herms & Mattson 1992). In doing so, understanding of the underlying mechanisms, including the dynamics of the emission of complex blends of plant volatiles, will be essential to understand how plants cope with the defence *vs.* growth trade-off. After all, the emission dynamics of plant volatiles including VOCs from flowers, and herbivore-induced plant volatiles are known to have diurnal and ontogenic patterns and, therefore, the effects of plant volatiles on interactions with community members from different trophic levels are expected to vary in time as well.

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Chapter 3

Reproductive escape: annual plant responds to butterfly eggs by accelerating seed production

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Abstract

Plants respond to insect herbivores with changes in physical and chemical traits, both locally and systemically, in leaves and flowers. Such phenotypic changes may influence the behaviour of every community member that interacts with the plant. Here, we address effects of plant responses to eggs and subsequent herbivory by caterpillars on plant-mediated interactions with pollinators, and consequences for plant fitness. Using a common garden set-up, we have investigated responses of *Brassica nigra* plants to herbivore exposure from egg deposition onwards throughout larval development. We quantified effects of infestation by the specialist *Pieris brassicae* on: 1. behaviour of pollinators; 2. volatile emission; 3. timing and number of seeds produced. Egg-deposition and folivory did not influence visitation by pollinators to plots of infested or control plants. Effects of herbivore infestation on both pollinator visitation and volatile emission were observed only at a later stage, when caterpillars were feeding on the flowers. Remarkably, before eggs had hatched, infested plants accelerated seed production. The caterpillars that developed from the eggs fed on flowers but not on seeds and, thus, seed production prior to herbivory on flowers safeguarded reproductive output. The results of this study show that early plant investments in reproduction can successfully prevent consumption of expensive reproductive tissues. By accelerating seed production, plants prevented consumption of flowers, and effectively defended themselves against the herbivores.

Keywords: bumblebees, egg-induced plant reproduction, florivory, flowers, folivory, herbivore-induced plant volatiles, seed production, syrphid flies.

Introduction

Plants respond to insect herbivores by changing physical and chemical traits (Kessler & Baldwin 2002, Erb *et al.* 2008, Dicke & Baldwin 2010). Induced responses can be expressed in leaves and flowers of damaged plants (Geervliet *et al.* 1994, Turlings & Fritzsche 1999, Dannon *et al.* 2010), and such phenotypic changes may influence the behaviour of every insect that interacts with the plant (Ohgushi 2005, Dicke 2009). Most attention has been paid, however, to the responses of arthropod predators and parasitoids foraging for host or prey (Vet & Dicke 1992, Kessler & Baldwin 2001, Dicke & Hilker 2003, D'Alessandro & Turlings 2006, Hilker & Meiners 2011). Yet, phenotypic responses by plants may also influence the behaviour of pollinating insects (Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011).

The enormous diversity of flower shapes, sizes, colours and odours has evolved among angiosperm plants to advertise the reward offered by the flowers to pollinating insects (Harder & Barrett 2006). Any phenotypic change in these traits may be associated by the pollinating insects with the quality of nectar and pollen offered as reward by the flowers (Weiss 1991, Raguso 2008, Rodriguez-Saona *et al.* 2011). Flower traits that change upon herbivore attack may positively or negatively influence foraging preferences of pollinators (Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011). Root-herbivory, for instance, positively influenced pollinator behaviour, rendering plants more attractive to flower visitors (Poveda *et al.* 2003). In contrast, herbivore damage to leaves and flowers negatively affected foraging preferences of pollinators, according to results of most studies performed so far (Kessler & Halitschke 2009). A negative impact on pollinator preference has not always resulted in negative impact on plant reproductive success. Plants may compensate for herbivory despite the negative effects of herbivore damage on the behaviour of pollinators (Lehtilä & Strauss 1997, Strauss *et al.* 2001, Steets *et al.* 2006).

In nature, plants that are flowering are exposed to pollinators and herbivores at the same time, and the interaction between plant and herbivore mostly starts at egg deposition (Hilker & Meiners 2011). In this context, we have used a common garden set-up, to investigate responses of *Brassica nigra* plants to herbivore exposure, throughout larval development, since egg deposition. We were interested in the overall effects on plant fitness. We quantified effects of herbivore infestation by the specialist *Pieris brassicae* (Fig. 1) on (1) behaviour of pollinators; (2) the emission of volatiles that may be used by pollinators, and (3) timing of seed set and number of seeds produced, including the contribution of day-active and night-active pollinators to seed production. As herbivores were exposed to naturally occurring predators and parasitoids, we also estimated dispersal and mortality of caterpillars during the experiments.

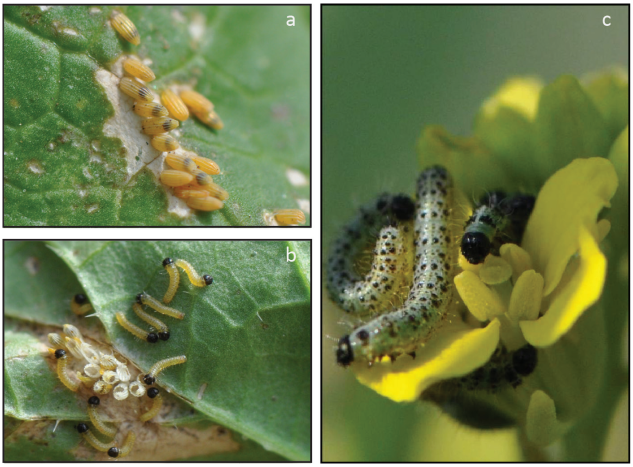


Fig. 1 *Pieris brassicae* eggs (a); *P. brassicae* larvae on a leaf (b) and on flowers (c) of *Brassica nigra* plants. Photo credits: Dani Lucas-Barbosa.

Material and methods

INSECTS AND PLANTS

Pieris brassicae L. (Lepidoptera: Pieridae) is a specialist herbivore of brassicaceous plant species. Female butterflies lay clusters of up to 100 eggs on the underside of leaves. When the eggs hatch, caterpillars will initially feed on the leaves of a flowering plant, but the second instar larvae will move to the flowers and become florivorous (Smallegange *et al.* 2007). The fifth instar larvae leave the plant to find a secluded pupation site. *Brassica nigra* L. (Brassicaceae) is considered to be an obligately out-crossing species (Conner & Neumeier 1995) and produce only hermaphroditic flowers. This plant species is a short-lived annual and a host plant of *P. brassicae.* In the Netherlands, *P. brassicae* caterpillars are regularly attacked by *Cotesia glomerata* L. (Hymenoptera: Braconidae) parasitoids.

In this study, *P. brassicae* was reared on *Brassica oleracea* var. *gemmifera* (Brussels sprouts) plants in a climate room (22 ± 1 °C, 50-70% r.h., L16:D8); the adults were provided with a 10% sucrose solution as food, in 5 mL vials with an opening in the centre of an artificial flower. Seeds of *B. nigra* were obtained from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands) from an early-flowering accession (CGN06619) and have been multiplied by exposing plants to open pollination in the surroundings of Wageningen. Seeds collected from 25 plants were mixed to obtain seed batches for the experimental plants. Potted *B. nigra* plants were reared outside on tables protected by insect screens, in a location close to the field site.

PLANT TREATMENT

Flowering plants with a few open flowers [growth stage 4.2, based on classification for *Brassica napus* (Harper & Berkenkamp 1975)], were infested with one egg clutch of *P. brassicae* by exposing plants to butterflies in an oviposition cage (100 cm × 70 cm × 82 cm). Flowers of the plant were covered with a mesh bag during exposure to butterflies to prevent flower visitation. The number of eggs on a plant was reduced to thirty by gently removing surplus eggs shortly after the plants had been removed from the oviposition cage. We infested four plants per day during four consecutive days, for each field trial. Egg-infested and control plants were transferred to the field right after egg deposition.

Effects of herbivore infestation on pollinator behaviour and consequences for plant fitness

Common garden experiment – field layout

Field layout consisted of 16 plots of *B. nigra* plants infested with *P. brassicae* and 16 control plots. Each plot (50 cm × 50 cm) was composed of 5 plants. The central plant of the plot was either infested with 30 *P. brassicae* eggs or was non-infested. The other four plants were all non-infested. Plots with an infested central plant are called "infested plots" and plots with a non-infested central plant are called "control plots" (Fig. 2). Equal numbers of control and infested plots were transplanted to the field on each of the four days. Plants that were infested on the same day were never planted in the same column or row in the field lay-out. Control and infested plots were planted alternately, and the distance between them was 1.5 m. Once plants had been transferred to the field, no attempt was made to prevent further infestation by any other herbivores. We carried out two serially repeated trials between May 23rd and July 23rd 2011, at an experimental field site in Wageningen, The Netherlands.

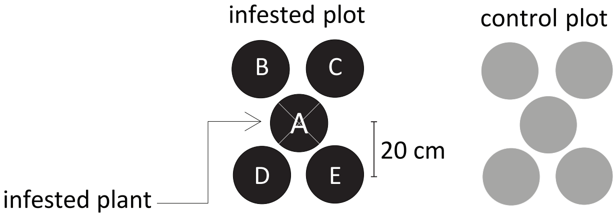


Fig. 2 Infested and control plots of plants. Each circle represents one plant, and only the plant in the centre of an infested plot was infested with *Pieris brassicae* eggs. The distance between the A-plant and any of the other plants of the same plot was 20 cm. Equivalent can be assumed for control plots of plants.

Dispersal and mortality of *Pieris brassicae*

The number of *P. brassicae* eggs on the egg-infested plants was counted at the end of egg development phase, as judged by darkening of the egg tips. If mortality of eggs or of the first instar larvae appeared to be higher than 50% in a given plot, the egg-infested plant of the plot received fifteen first-instar caterpillars (*i.e.* 50% of initial number of eggs) obtained from the insect culture. To keep track of the actual amount and location of herbivore activity, we estimated dispersal and mortality of caterpillars by counting the number of individuals present on each of the five plants per plot, for each of the 16 infested plots in both trials. Number of caterpillars and their position on the plant were scored every other day. Caterpillar position was recorded in three categories, on (1) a leaf, (2) a flower/bud, or (3) the stem. At the end of the experiments, when plants were harvested, caterpillars that were found on the plants were placed in Petri dishes, to assess whether they had been attacked by a parasitoid. These caterpillars were subsequently kept in a greenhouse compartment (22 ± 1 °C, 50-70% r.h., L16:D8) and were fed with *B. nigra* flowers/leaves until pupation or until parasitoid larvae emerged from the caterpillar. Parasitism frequency was calculated as the number of parasitized caterpillars divided by the sum of hatched herbivore eggs and inoculated caterpillars.

Effects of herbivore infestation on visitation by pollinators

Pollinator visitation to infested and control plots was observed throughout *P. brassicae* egg and larval development. We observed the behaviour of pollinators at six time points spaced between the moment when plants were infested with eggs until the time at which larvae had reached the final larval instar. The six time points were classified into three groups, as follows: (a) 24 h and 144 h after egg-deposition (egg-infestation only); (b) 24 h and 120 h after caterpillars had hatched (leaf-feeding only); and (c) 216 h and 312 h after caterpillars had hatched (on-going flower-feeding). When a pollinator had contact with a flower, we recorded the pollinator's family [for Apidae, we distinguished bumblebees, *Apis mellifera* (honeybees) and 'other Apidae'], number of flowers visited, and time spent per flower. If other pollinator individuals happened to enter the plot under observation, we recorded only their visitation and family. 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. Each plot was observed for 10 min, and visitations were recorded 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). Observation of pollinator visitation to infested and control plots was performed alternately and a maximum of 8 plots were observed within a day. Observations were carried out whenever weather conditions were suitable (17-25 °C – wind speed ≤ 6 m sec $^{-1}$), between 9 am and 4 pm. We did not record number of flowers visited by pollinators for the first time point, *i.e.* when infested plants carried only eggs, for trial 1.

Effects of herbivore infestation on plant growth and seed production

In the same field set-up as described above we investigated whether treatment affected growth and reproduction of *B. nigra* plants. At three time points per week, we scored which plants started to produce seeds. When caterpillars had reached the last larval instar, plants were harvested and siliques separated from shoots. We quantified seed production as a measure of reproductive success of *B. nigra* plants infested with *P. brassicae* compared with that of control plants. Seeds produced by infested and control plants were counted and weighed. When the total number of seeds was smaller than one hundred for an individual plant, all seeds were counted. When number of seeds was greater than one hundred seeds, the total number of seeds was estimated as total seed weight divided by the weight of one hundred seeds of that plant. To evaluate plant growth, we measured aboveground biomass of infested and control plants. Plant shoots were dried overnight (105°C), and dry biomass was determined.

Contribution of day- and night-active pollinators to seed set of *Brassica nigra* plants

The contribution of day- and night-active pollinators to seed-set of *B. nigra* plants was investigated in the same location as described above. The experiment was carried out between July 25th and August 19th 2011. During this period in The Netherlands, the sun rises around 6:30 am and sunset is around 8:00 pm. Field layout was similar to the one used for the trials described above. In total, twenty-four plots of five plants each were transplanted to the field; half of the plots were infested with *P. brassicae* eggs (infestation procedure described above) and half were non-infested control plots. Plants in twelve plots were exposed to pollinators during daytime (7:00-7:30 am – 7:00-7:30 pm), and covered with a tent (BugDorm, 95 cm × 95 cm × 190 cm, white fine mesh in polyester 100 × 80 square/inch) during the night (7:00-7:30 pm – 7:00-7:30 am). The other 12 plots were exposed to pollinators during the night, and covered with a tent during daytime. Twenty-seven days after egg infestation, plants were harvested and siliques separated from shoots. Seed production and shoot dry biomass were quantified as described in the previous paragraph.

Headspace collection of plant volatiles in the field and analysis by GC-MS

We collected plant volatiles from aerial parts of infested and control plants. Headspace samples from egg-infested, leaf-infested, flower-infested, and similarly aged non-infested control plants for each of the three groups were collected between July 25th and September 16th of 2011, whenever weather conditions were suitable, and in the same location, as described above. Aerial parts of the central plants of infested and control plots were enclosed with an oven bag (Toppits® Brat-Schlauch; polyester; 32 cm × 32 cm × 100 cm). Bags were closed around the stem and above the flowers with a strip of bag material. We pumped air from the environment into the bag at a flow rate of 300 mL min^{-1} (224-PCMTX8, air-sampling pump Deluxe, Dorset, UK; equipped with an inlet protection filter) by inserting Teflon tubing through an opening in the upper part of the bag. By inserting a second Teflon tube at the opening of each bag, and connecting it to a glass tube filled with about

90 mg of Tenax-TA 25/30 mesh (Alltech, Breda, NL), air was sucked out and headspace volatiles were collected in the glass tube filled with Tenax for 1.5 h at a flow rate of 250 mL min^{-1} (224-PCMTX8, air-sampling pump Deluxe, Dorset, UK). Bags were discarded after use. Plant volatiles of egg-infested, leaf-infested, flower-infested, and control plants of the same stages were collected from in total 16 infested and 16 control plants, over 2 trials.

Headspace samples were analysed in a gas chromatograph with a thermodesorption unit (GC) (6890 series, Agilent, Santa Clara, USA) connected to a mass spectrometer (MS) (5973 series, Agilent, Santa Clara, USA). Collected volatiles were desorbed from the Tenax in a thermodesorption trap unit (Gerstel, Mülheim, Germany) by heating from 25 °C to 250°C (5 min hold) at a rate of 60 °C min^{-1} in splitless mode. Released compounds were focused in a cold trap (ID 1.80 mm) filled with glass beads (d 0.75-1.00 mm) at a temperature of -50 °C. By flash heating of the cold trap to 250 °C at 12 °C sec^{-1}, volatiles were transferred to the analytical column (60 m × 0.25 mm ID, 0.25 μm film thickness, DB-5, J&W, Folsom, USA). Oven temperature programme started at 50 °C (1 min hold) and rose at a rate of 20 °C min^{-1} to 100 °C, subsequently increased at a rate of 4 °C min^{-1} to 280 °C (1.5 min hold) and finally rose up to 300 °C at a rate of 10 °C min^{-1}. Column effluent was ionized by electron impact ionization at 70 eV. Mass scanning was carried out from 40 to 300 *m/z* with 5.36 scans sec^{-1}. Compounds were identified by comparison of mass spectra with those of NIST, Wiley libraries and the Wageningen Mass Spectral Database of Natural Products. Identity was confirmed by comparison of retention index described in the literature and the respective index calculated during this study. Compounds were quantified when detected in at least 50% of infested or control plants, for each of the 3 stages.

Results

POLLINATOR BEHAVIOUR

Behaviour of day-active pollinators in infested and control plots was quantified when plants carried only eggs, and when caterpillars were primarily feeding on leaves or flowers. Time spent per flower, mean number of flowers visited and mean number of flower visitors to infested and control plots were quantified for the main groups of flower visitors observed: bumblebees, honey bees, other Apidae, Syrphidae and Lepidoptera. Among the syrphid flies, *Eristalis tenax* was the main species observed, and *Bombus lapidarius* was the most abundant bumblebee species (Fig. 3). The cabbage white butterflies *Pieris rapae* and *P. brassicae* were the main lepidopterans observed visiting flowers of *B. nigra* plants, but their numbers were not large enough to be statistically analysed.

Fig. 3 Main flower visitors of *Brassica nigra* observed during this study: a) *Bombus lapidarius;* b) *Eristalis tenax;* c) *Apis mellifera*. Photo credits: Dani Lucas-Barbosa.

Bumblebees were by far the most abundant group of pollinator insects observed throughout trial 1 (results not shown), but were no longer flying during trial 2. In trial 1, flowers of *B. nigra* were more frequently visited by bumblebees than by honeybees (ANOVA, Tukey post-hoc test, *P* = 0.025) and syrphid flies (ANOVA, Tukey post-hoc test, *P* = 0.001), during the flower-infestation period. Also during the leaf-infestation period, bumblebees visited a larger number of *B. nigra* flowers than did syrphid flies (ANOVA, Tukey post-hoc test, *P* = 0.037).

Neither during the development of the butterfly eggs, nor during the time when the caterpillars were feeding on the leaves, did we observe effects of herbivore treatment on pollinator behaviour. However, effects of herbivory on pollinator behaviour were observed at the latest stage of infestation, when caterpillars were feeding on the flowers of infested plants. In trial 1, florivory influenced the behaviour of bumblebees and syrphid flies in different ways. When most caterpillars were feeding on flowers, bumblebees visited more flowers of infested plots than flowers of control plots (Fig. 4, Student's *t*-test, *P* = 0.048). Syrphid flies spent more time on flowers of control plots than on flowers of infested plots (Fig. 4, Student's *t*-test, *P* = 0.034). Behaviour of honeybees was not influenced by herbivore infestation in either of the two trials. In trial 2, infested plots received as many visits as control plots by all insects observed (See supporting information in Appendix B, Fig. B1). No overall effect of treatment was observed (Appendix B, Tables B1 and B2, repeated measures ANOVA). Number of pollinators increased throughout the field season, and, consequently, a larger number of flowers were visited later in the season (Appendix B, Tables B1 and B2).

PLANT VOLATILE EMISSION

Volatile blends produced by infested and control plants changed quantitatively and qualitatively throughout the flowering stage and during seed development (Table 1 and Appendix B, Fig. B2). A Projection to Latent Structures Discriminant Analysis (PLS-

DA) (SIMCA P+ 12.0, Umetrics AB, Umeå, Sweden) including volatiles emitted by egg-infested, leaf-infested, flower-infested, as well as control plants at the same developmental plant stages resulted in a model with two significant principal components (explained variance 0.047) that separated the samples of the three plant developmental stages to a large extent, regardless of the herbivore treatment (Appendix B, Fig. B2). Monoterpenoids were characteristic of headspace samples of leaf-infested, flower-infested plants and their respective control plants, but were not characteristic of headspace samples of egg-infested plants and their respective control plants (Table B3). Irrespectively of the treatment, the composition of the volatile blend emitted by plants quantitatively and qualitatively changed throughout the flowering stage, and these differences were more pronounced than the differences between treatments (Table B3). The total volatile emission by flower-infested plants was on average lower when compared with the respective control plants (Wilcoxon's signed rank test, *P* = 0.001). This was not the case when volatile blends from leaf-infested (Wilcoxon's signed rank test, *P* = 0.346) and egg-infested plants (Wilcoxon's signed rank test, *P* = 0.052) were compared with blends from control plants of the same developmental stages.

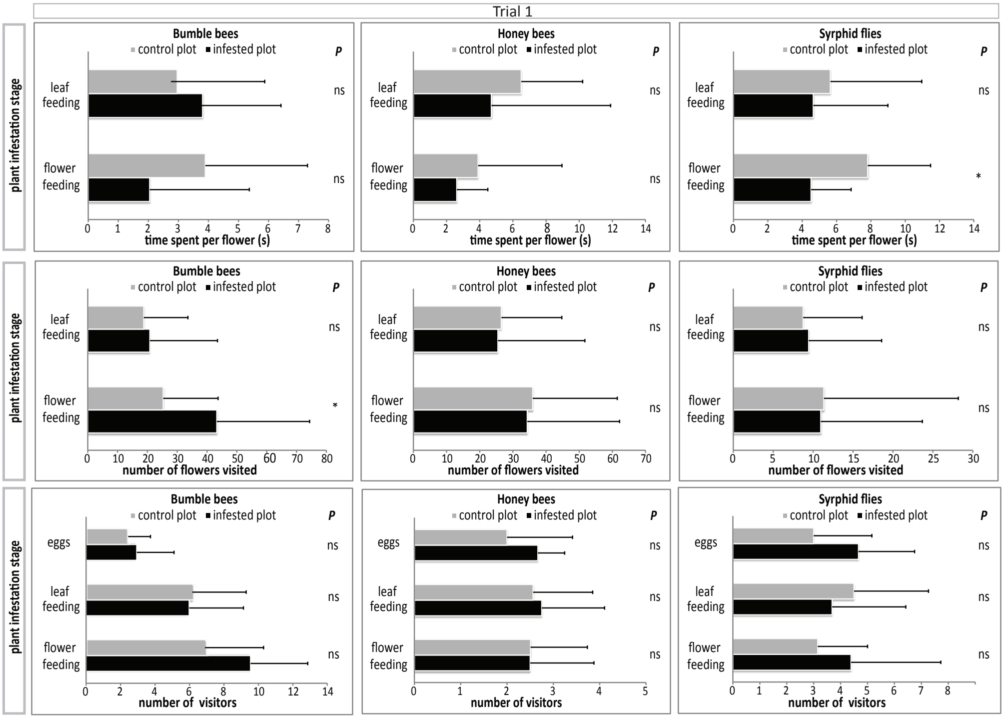


Fig. 4 Time spent per flower (s) (mean + SD), number of flowers visited (mean + SD) and number of flower visitors per plot (mean + SD) to *Pieris brassicae*-infested and control *Brassica nigra* plots, per 10 min of observation. Visitation by bumblebees, honeybees and syrphid flies to infested and control plots were recorded when plants carried eggs and when most herbivores were feeding on leaves or flowers. Results are presented for trial 1. Pairwise comparison of treatments with independent *t*-test (α = 0.05; *, P ≤ 0.05; ns, not significant).

Dispersal and mortality of *Pieris brassicae*

The *P. brassicae* eggs hatched after about 10 days; mean temperatures during trials 1 and 2 were 15 °C and 17 °C, respectively. Survival of eggs per plot was higher during trial 1 (74 %) than during trial 2 (27 %) (Fig. 5). When eggs hatched, caterpillars initially fed on the leaves on which the eggs had been laid, and within 96 h the caterpillars were found feeding on flowers (Fig. 6). Once caterpillars reached flowers they also dispersed to neighbouring plants of the same plot (Fig. 6).

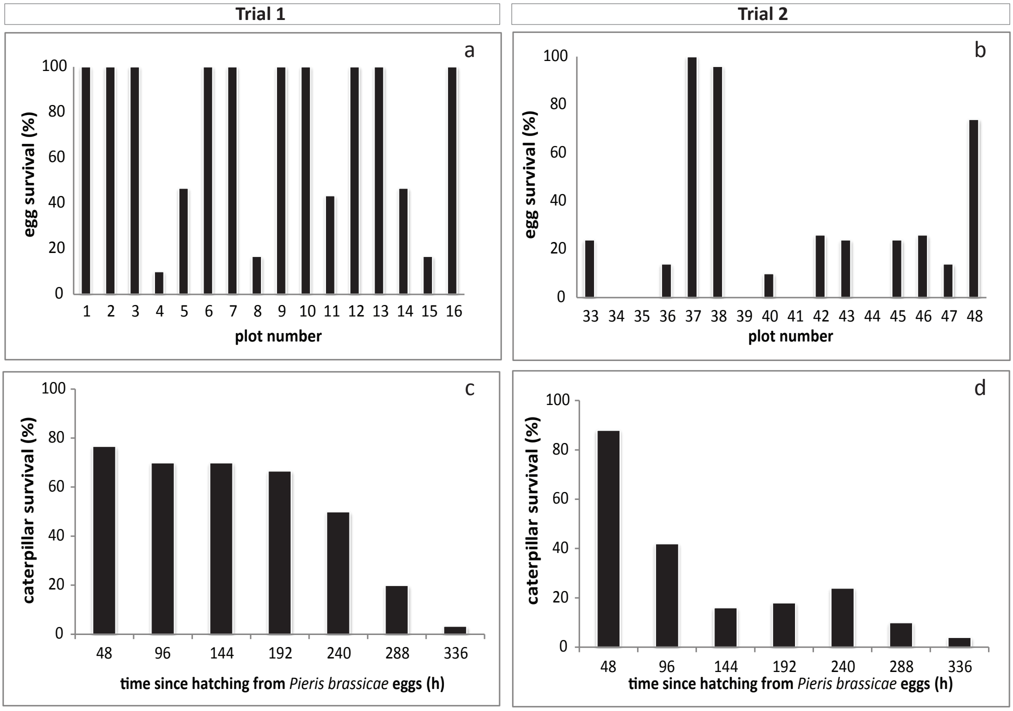


Fig. 5 Survival of eggs (%) (a, b) per plot and survival of *Pieris brassicae* caterpillars (%) (c, d) over time since herbivores hatch from the eggs, for trials 1 and 2.

Caterpillar survival was also higher during trial 1 (51%) than during trial 2 (29%) (Fig. 5). Eggs and early larval instars of *P. brassicae* were preyed upon mainly by ladybird beetles (Coccinellidae), whereas later larval instars were often predated by yellow jackets (*Vespula* spp.; D. Lucas-Barbosa, personal observation). Of the caterpillars collected at the end of both trials, less than 1% was parasitized.

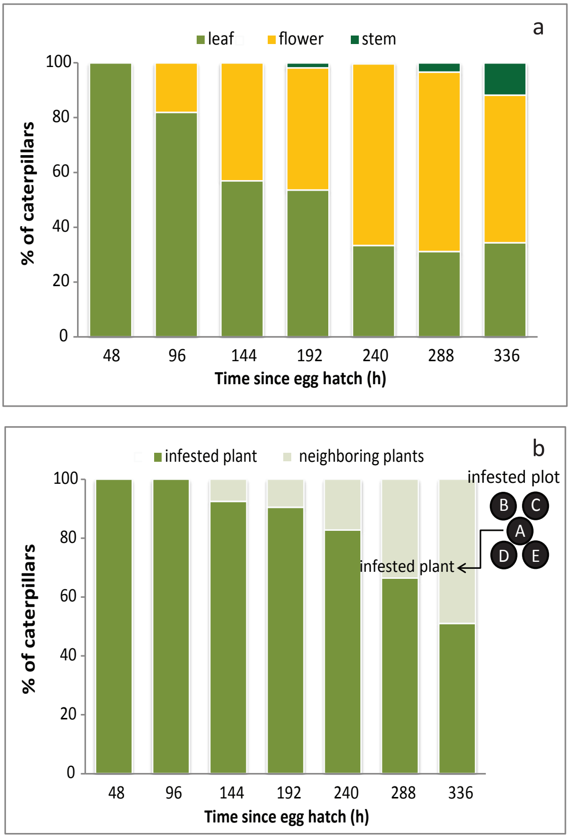
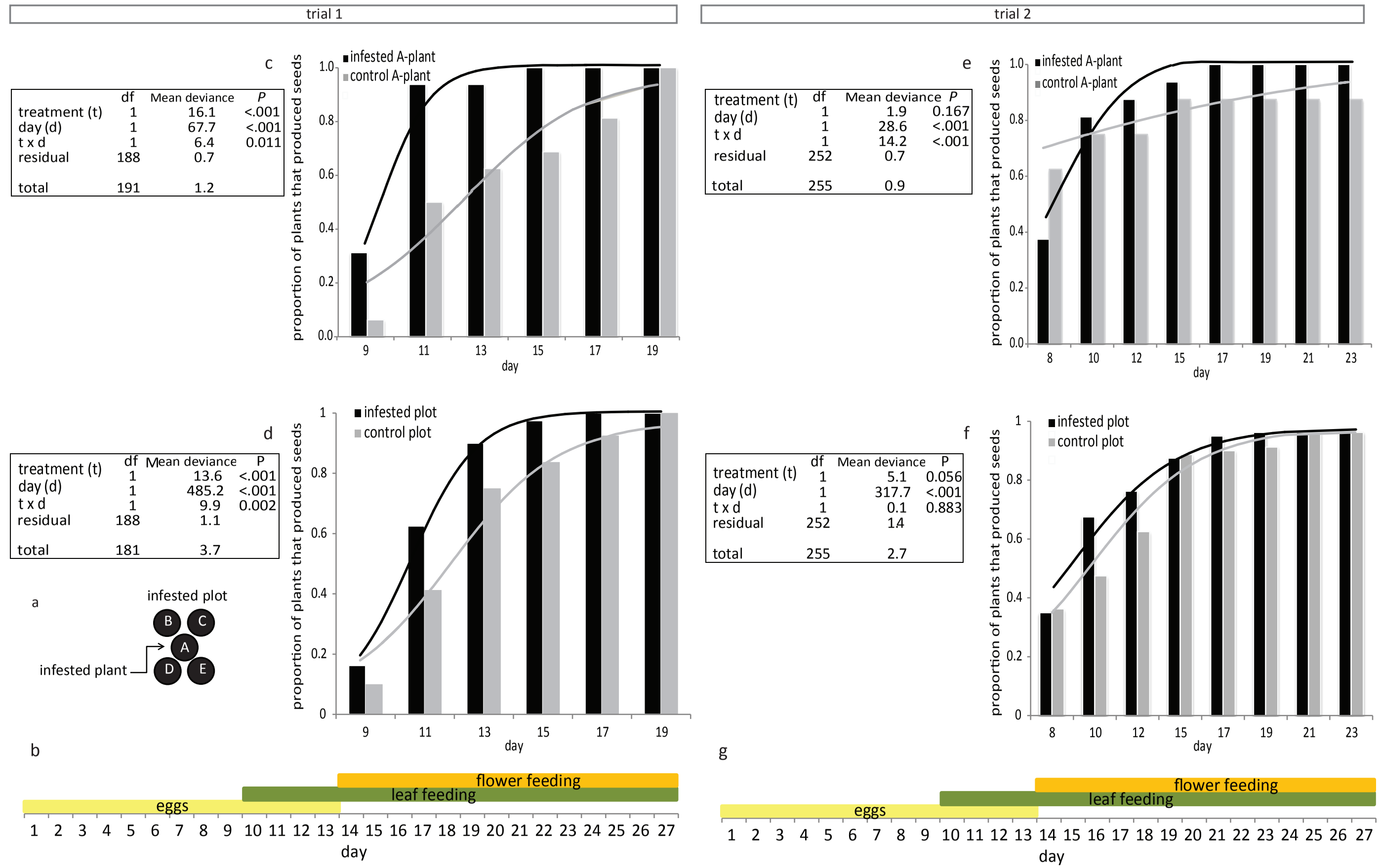


Fig. 6 Site of feeding by *Pieris brassicae* caterpillars within one plant, and within the plot. Percentage of caterpillars found on a leaf, flower or stem, over time since caterpillars hatch from eggs (a). Percentage of caterpillars found on the originally egg-infested A-plant or on one of the neighbouring plants (B, C, D and E) (b). Results are shown for trial 1.

PLANT GROWTH AND SEED PRODUCTION

Brassica nigra plants that had been infested with *P. brassicae* eggs at the start of the experiment produced seeds sooner than control plants, at both plant and plot levels (Fig. 7). Elongation of siliques and seed formation was already observed at a very early stage of herbivore development in the infested plots, *i.e.* when plants still carried eggs or when caterpillars had only just hatched (see Fig. 7b). For instance, in trial 1 we observed that 11 days after plants had been infested with *P. brassicae* eggs, a larger number of infested plants had produced seeds when compared to non-infested plants (Fig. 7, chi-square test, $P_{\text{A-plant}} = 0.001$ and $P_{\text{plot}} = 0.007$). Moreover, in trial 1 infested plants produced more seeds than control plants, at both plant and plot levels (Fig. 8, Student's *t*-test, $P_{\text{A-plant}} = 0.036$ and $P_{\text{plot}} = 0.012$). In trial 2, infested plants and control plants produced equal numbers of seeds (Fig. 8, Student's *t*-test, $P_{\text{A-plant}} = 0.860$ and $P_{\text{plot}} = 0.740$).

Infested plants produced as many seeds as did control plants, irrespective of having been exposed to pollinators during night or day. Plants that were exposed to night-active pollinators produced as many seeds as did plants that were exposed to day-active pollinators (results not shown). Shoot dry weights of infested and control plants were similar for all experiments carried out during this study (results not shown).



c

	df	Mean deviance	*P*
treatment (t)	1	16.1	<.001
day (d)	1	67.7	<.001
t x d	1	6.4	0.011
residual	188	0.7	
total	191	1.2	

d

	df	Mean deviance	P
treatment (t)	1	13.6	<.001
day (d)	1	485.2	<.001
t x d	1	9.9	0.002
residual	188	1.1	
total	181	3.7	

e

	df	Mean deviance	*P*
treatment (t)	1	1.9	0.167
day (d)	1	28.6	<.001
t x d	1	14.2	<.001
residual	252	0.7	
total	255	0.9	

f

	df	Mean deviance	P
treatment (t)	1	5.1	0.056
day (d)	1	317.7	<.001
t x d	1	0.1	0.883
residual	252	14	
total	255	2.7	

Fig. 7 Proportion of infested and control *Brassica nigra* plants that produced seeds, throughout trials 1 and 2, at plant and plot levels; Schematic representation of a field plot; only the centre A-plant was infested with *Pieris brassicae* eggs (a); Scale shows length of the experiment and period during which plants were exposed to eggs, leaf feeding and flower feeding by caterpillars for trials 1 (b) and 2 (g); Proportion of infested- and control A-plants that produced seeds as a function of time for trials 1 (c) and 2 (e); Proportion of infested and control plots that produced seeds as a function of time for trials 1 (d) and 2 (f). Logistic regression and accumulated analysis of deviance is shown.

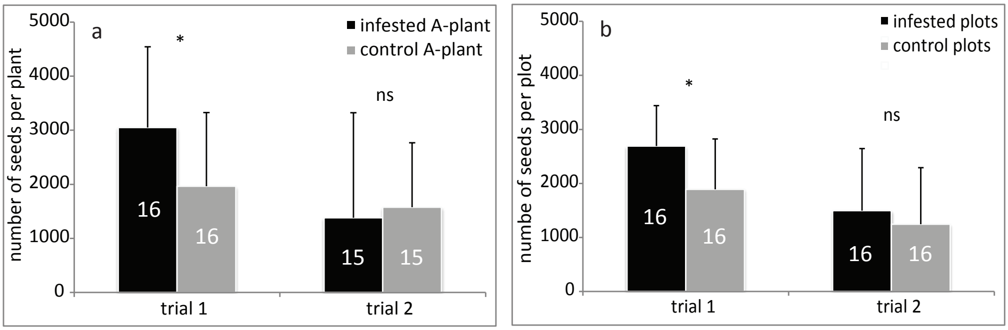


Fig. 8 Number of seeds produced (mean + SD) by *Pieris brassicae* infested and control *Brassica nigra* plants at plant (a) and plot levels (b) for trials 1 and 2. Pairwise comparison of treatments with independent *t*-tests ($\alpha = 0.05$; *, $P \leq 0.05$; ns, not significant). Number of plants (a) and plots (b) is shown in the bars. Each plot is composed of 5 plants. The central plant of the plot was either infested with 30 *P. brassicae* eggs or was non-infested; the other four plants were all non-infested.

Discussion

Our data show that *B. nigra* plants compensated for herbivory in terms of seed production, despite the changes observed in pollinator behaviour. Effects of herbivore infestation on pollinator behaviour were observed only when caterpillars were feeding on flowers of plants in infested plots. Egg-deposition and folivory did not influence visitation by pollinators of infested plots compared with control plots of plants.

Flower-infestation influenced the behaviour of various pollinators in different ways. Bumblebees visited more flowers in infested plots than in control plots. Syrphid flies, however, spent less time on flowers of infested plots than on flowers of control plots. Comparable to our observations regarding pollinator behaviour, effects of herbivore infestation on volatile emission were also only observed in the final stage of the plant-herbivore interaction, *i.e.* when caterpillars were feeding on the flowers. We collected headspace volatiles from egg-infested, leaf-infested, flower-infested, and control plants at the same stages, under field conditions, and observed that volatile emission by flower-infested plants was on average lower when compared with the respective control plants. Most studies investigating effects of herbivore infestation on the behaviour of pollinators have concluded that folivory and florivory mainly repel pollinators (Kessler & Halitschke 2009); *i.e.* pollinators were less attracted to herbivore-infested plants when compared with control plants. Repellence of pollinators was in some instances associated with increased volatile emission (Kessler & Halitschke 2009, Zangerl & Berenbaum 2009). Indeed, plants in the vegetative stage usually respond to herbivory by increasing emission of volatiles (Mumm & Dicke 2010). Results of our study indicate that volatile emission can also be reduced in response to herbivore infestation. Thus, plants can increase or decrease volatile emission in response to herbivory, perhaps depending on their phenological state (Hare

2010, Diezel *et al.* 2011). Herbivore egg deposition can also result in reduced phytochemical responses, including reduced volatile emission, according to the studies performed so far (Bruessow *et al.* 2010, Fatouros *et al.* 2012). Pollination too may result in reduced emission of floral volatiles (Rodriguez-Saona *et al.* 2011). Irrespective of whether phytochemical responses to pollination and insect herbivores are increased or decreased, insects that interact with the plant may respond to these changes. The net effects on phytochemical responses, and ultimately plant fitness, will be influenced by plant-mediated interactions between herbivore and pollinators, starting from egg deposition by adult herbivores.

Surprisingly, in this study, *B. nigra* plants responded to egg deposition with accelerated seed production. Herbivore-infested *B. nigra* plants produced seeds sooner than did non-infested control plants. It is remarkable that this already occurred before the herbivore eggs had hatched. Eggs are the beginning of a new herbivore generation, and plants are known to respond to egg-deposition (a) with responses that kill herbivorous larvae while developing in the eggs (Hilker & Meiners 2011), (b) with changes that influence the performance of herbivorous larvae (Bruessow *et al.* 2010, Beyaert *et al.* 2012), and (c) by inducing chemical cues that are exploited by egg parasitoids to locate their hosts and by butterflies during host plant selection (Blaakmeer *et al.* 1994, Fatouros *et al.* 2005). Our results show a novel response of plants to herbivore egg-deposition. Early seed production in response to egg-deposition by *P. brassicae* is likely to be advantageous for *B. nigra*. Caterpillars of *P. brassicae* are voracious, specialist leaf and flower feeders of *B. nigra* plants (Smallegange *et al.* 2007) and these plants considerably tolerate damage to leaves (Blatt *et al.* 2008). *Pieris brassicae* caterpillars actually prefer to feed on flowers of *B. nigra* plants rather than on leaves, consuming flowers entirely and in large numbers (Smallegange *et al.* 2007). These caterpillars do not feed, however, on seeds of *B. nigra* plants (D. Lucas-Barbosa, personal observation). Thus, by accelerating seed production, *B. nigra* plants safeguard their reproduction before the caterpillars can consume the flowers.

In this study, accelerated seed production also resulted in compensation for herbivory, as infested plants produced as many seeds as non-infested control plants. Although plants compensated for herbivory, this was probably not mediated by modified interactions with pollinators. *Brassica nigra* is considered to be an obligately outcrossing species (Conner & Neumeier 1995), and thus dependent on pollinators for reproduction. The observation that herbivore-infested plants produced seeds sooner and in similar or larger numbers than non-infested control plants, suggests that plots of infested plants received more visitation by pollinators than non-infested plots. Yet, we do not expect that the compensation in terms of seed production by the infested over control plants, during this study, could be the result of an efficient pollination service provided by bumblebees. Bumblebees indeed visited more flowers in infested plots than in control plots, but only during a later stage, when caterpillars were feeding on the flowers. An enhanced seed-production rate was observed in the beginning of the experiments, when plants carried only eggs, and at this time point, egg-infested plots did not receive more visitation by bumble-bees than non-infested plots. This indicates that plant-mediated interactions between herbivores and pollinators do not explain how *B. nigra* plants compensated for herbivory.

The accelerated seed production recorded in trial 1 was also visible in trial 2, albeit that the effect was attenuated when compared to results from trial 1. The observation that egg mortality in trial 2 was much higher than in trial 1 reinforces our conclusion that plant responses to egg-deposition triggered the accelerated reproduction in this system. Plants may compensate for herbivory by investing in self-pollination over outcrossing. *Brassica nigra* plants produce only hermaphroditic flowers; thus we hypothesize that under stress, self-reproduction is favoured *via* autogamy. Plants can compensate for herbivory despite negative effects on the behaviour of obligate pollinators (Junker & Bluthgen 2010), and there is indeed evidence that direct plant responses to herbivore damage can increase plant reproductive success (Lehtilä & Strauss 1997, Steets *et al.* 2006, Wise *et al.* 2008, Penet *et al.* 2009, Wise & Hebert 2010). Our data show that even before any herbivore damage had occurred, *B. nigra* plants responded to egg-deposition and accelerated seed production.

Interestingly, early seed production was observed not only by the plants that were infested with *P. brassicae* eggs, but also by the other four plants of the same plot that did not receive egg-deposition. The effects on the other 4 plants of the same plot may have been mediated either by pollinator attraction or by plant-plant communication. We did not observe that visitation rates by pollinators were higher in egg-infested plots than in control plots. Plant-plant communication may have occurred above and belowground (Heil & Karban 2010). Whether this has played a role remains to be investigated.

The annual plant *B. nigra* invested in seed production in response to egg deposition by *P. brassicae,* and not in increased volatile emission as typically observed after herbivory for plants in the vegetative stage. Our data show that accelerated investments in reproduction can successfully prevent consumption of expensive reproductive tissues and enable plants to compensate for herbivory. In other words, by investing in reproduction before herbivores attack reproductive organs, plants can effectively defend themselves against herbivorous insects.

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Chapter 4

Folivory affects composition of nectar, floral odour and modifies pollinator behaviour

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Abstract

Herbivory induces changes in plants that influence the associated insect community. The present study addresses the potential trade-off between plant phytochemical responses to insect herbivory and interactions with pollinators. We used a multidisciplinary approach and have combined field and greenhouse experiments to investigate effects of herbivory in plant volatile emission, nectar production, and pollinator behaviour, when *Pieris brassicae* caterpillars were allowed to feed only on the leaves *Brassica nigra* plants. Interestingly, volatile emission by flowers changed upon feeding by herbivores on the leaves, whereas, remarkably, volatile emission by leaves did not significantly differ between infested and non-infested flowering plants. The frequency of flower visits by pollinators was generally not influenced by herbivory, but the duration of visits by honeybees and butterflies was negatively affected by herbivore damage to leaves. Shorter duration of pollinator visits could be beneficial for a plant, because it sustains pollen transfer between flowers while reducing nectar consumption per visit. Thus, no trade-off between herbivore-induced plant responses and pollination was evident. The effects of herbivore-induced plant responses on pollinator behaviour underpin the importance of including ecological factors, such as herbivore infestation, in studies of the ecology of plant pollination.

Keywords: *Brassica nigra* (black mustard), flower visitors, herbivore-induced plant volatiles, herbivory, honeybees, syrphid flies.

Introduction

Plants interact with a wide variety of herbivores, pathogens, carnivores and pollinators (Schoonhoven *et al.* 2005). When herbivorous insects feed from a plant, the chemical composition, including volatile emission, can change and thereby affect behavioural responses of insects in the community. Some herbivorous species avoid damaged plants, whereas carnivorous insects exploit the herbivore-induced cues to find plants harbouring hosts or prey (Dicke & Baldwin 2010). These dynamic interactions have been studied extensively for vegetative plants (Karban & Baldwin 1997, Schoonhoven *et al.* 2005, Mumm & Dicke 2010). However, a full understanding of such interactions requires that the effect on plant reproduction is investigated. Induced plant responses can be costly due to allocation of resources or due to ecological costs (Dicke & Sabelis 1989, Simms 1992, Karban & Baldwin 1997, Strauss *et al.* 2002). Allocation costs result from diverting nutrients to defence responses rather than to growth or reproduction (Herms & Mattson 1992). Ecological costs arise from interactions with community members that have a negative impact on plant fitness as a consequence of the herbivore-induced response (Strauss & Murch 2004). For instance, the induction of defence in plant leaves might interfere with the attraction of pollinators to the flowers (Kessler *et al.* 2011, Lucas-Barbosa *et al.* 2011). In this study, we have investigated how foliar herbivory affects plant–pollinator interactions.

About two thirds of all plant species depend on pollination by insects for seed production (Myers 1996, Schoonhoven *et al.* 2005, Klein *et al.* 2007, Ollerton *et al.* 2011). Pollinator visitation is influenced by the quality and quantity of pollinator rewards, i.e. pollen and nectar and by traits that may be associated with reward quality such as number, size, odour and colour of flowers (Harder & Barrett 2006). Plants that offer nectar as reward and depend on pollination for reproduction should attract insects by producing nectar in amounts just large enough to reward an insect pollinator, but not enough to satisfy the needs of the visiting insect. An ideal pollinator would be an insect that lands on the flower, collects a sufficient amount of pollen and visits many other plants to which it donates the pollen resulting in fertilization. The more plants it visits, the more pollen is spread (Klinkhamer & Dejong 1993, Schoonhoven *et al.* 2005). Besides quantity, also toxins in nectar can limit nectar feeding and, therefore, increase the number of flower visits and decrease the duration per visit (Kessler & Baldwin 2007, Irwin & Adler 2008).

Insect herbivory induces many changes in a plant. It can affect the development and reproduction of plants directly by, for instance, removing reproductive tissue, or indirectly through reduced pollination (Lehtilä & Strauss 1997, Strauss *et al.* 2001, Schoonhoven *et al.* 2005, Kessler & Halitschke 2009, Cardel & Koptur 2010, Danderson & Molano-Flores 2010). Herbivory in an early stage reduces the photosynthetic capacity of the plant and may result in smaller plants with a delayed and shorter flowering period (Zangerl *et al.* 2002, Poveda *et al.* 2003). Other observed effects of insect herbivory on plants are changes in the production of pollen and nectar, changes in the quality of nectar, reduced seed and fruit production and changes in number and morphology of flowers (Lehtilä & Strauss

1997, Hamback 2001, Oguro & Sakai 2009). Herbivory can also affect floral chemistry. For example, leaf herbivory in tobacco plants increased alkaloid concentration in the nectar (Adler *et al.* 2006), whereas other studies have shown increases in defence compounds such as nicotine and glucosinolates in flower tissues (Euler & Baldwin 1996, Ohnmeiss & Baldwin 2000, Strauss *et al.* 2004). Also, floral volatile emission can change upon herbivore feeding, even when the damage is inflicted to leaves. Herbivory can increase or decrease volatile emission depending on herbivore species and feeding mode (Effmert *et al.* 2008, Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011, Pareja *et al.* 2012). As a result, herbivory may affect pollinator visitation (reviewed in Strauss & Murch 2004, Lucas-Barbosa *et al.* 2011). Depending on the species involved, herbivory may either decrease (Lehtilä & Strauss 1997, Kessler *et al.* 2011), or increase pollinator visitation (Poveda *et al.* 2005).

The present study addresses the potential trade-off between responses to herbivory and attraction of pollinators in the annual wild plant *Brassica nigra.* We addressed the following questions: 1) does folivory by *Pieris brassicae* influence number and duration of flower visits by different pollinators? 2) does insect folivory change volatile emission from leaves as well as from flowers? To study whether nectar rewards play a role in changing flower visitation after herbivory we asked: 3) whether nectar quantity and quality, more specifically glucosinolate levels and sugar concentration, change upon feeding damage by herbivores.

Materials and methods

Plant and Insects

Black mustard, *Brassica nigra* L. (Brassicaceae), plants were grown from seeds collected in the field from open-pollinated plants of *B. nigra* accession CGN06619 (obtained from the Centre for Genetic Resources, Wageningen, NL). Plants were grown in a greenhouse compartment (22 ± 2 °C, 60 ± 10% r.h, 16L:8D). Flowering plants of approximately 7 weeks old, with many open flowers [growth stage 4.2 - (Harper & Berkenkamp 1975) were used for the experiments. Flowers of *B. nigra* remain open for 5 days, and new flowers open daily.

Stock colonies of *Pieris rapae* (L.) and *P. brassicae* (Lepidoptera: Pieridae) were maintained on Brussels sprouts plants (*Brassica oleracea* var *gemmifera* L.) in a climate room (21 ± 1 °C, 60 ± 10% r.h, 16L:8D). Adult butterflies were provided with 10% sugar water and Brussels sprouts plants to oviposit on. Pupae of the marmelade hoverfly, *Episyrphus balteatus* (De Geer, 1776) (Diptera: Syrphidae), were obtained from Koppert Biological Systems (product name: Syrphidend). The flies were fed with 10% sugar water and pollen from *B. nigra*. Flies of 2- to 7-days-old were used in experiments. A small colony of honey bees (*Apis mellifera* L. Hymenoptera: Apidae) was provided by Inbuzz (Wageningen, NL) and consisted of three frames with brood of all stages plus the laying queen. The bees were provided with *B. nigra* plants and 10% sugar water in artificial flowers.

In the field experiment, visitation by pollinators from naturally occurring populations of honey bees, bumble bees, solitary bees and syrphid flies was monitored.

Plant Treatments

For all experiments, each *Brassica nigra* plant was infested with 100 second instar *P. brassicae* larvae, spread over two leaves, and allowed to feed for 48 hours. To prevent caterpillars from moving to flowers, cotton wool was placed around the petiole leaf stalk or the caterpillars were confined to clip cages. When plants were tested, caterpillars had consumed about 60 % of the leaves where they were placed. Control plants were of the same batch of plants, of the same age and similar in height and number of flowers, but were not infested with herbivores.

Headspace Collection of Plant volatiles and Analysis by GC-MS

To investigate whether flowering *B. nigra* plants respond locally and systemically to herbivore infestation, we collected plant volatiles from leaves and flowers of infested and non-infested control plants. Experiments were performed in a greenhouse compartment (22 ± 2 °C, 60 ± 10 % r.h. L16:D8). Either leaves or flowers of infested and control plants were enclosed in an oven bag (Toppits® Brat-Schlauch, polyester; 32 cm x 32 cm x 70 cm; Toppits, Minden, Germany). A strip of bag material wrapped around the stem and above or below the inflorescence was used to close the bag. Synthetic air was flushed through the bag at a flow rate of 300 mL min^{-1} (224-PCMTX8, air-sampling pump Deluxe, Dorset, UK; equipped with an inlet protection filter) by inserting Teflon tubing through an opening in the upper part of the bag. Air was sucked out and headspace volatiles were collected in the glass tube filled with Tenax for 1.5 h at a flow rate of 250 mL min^{-1} through a second Teflon tube at the opening of each bag, and connecting it to a glass tube filled with about 90 mg of Tenax-TA 25/30 mesh (Grace-Alltech), Bags were discarded after use. Plant volatiles of in total 10 infested and 10 control plants were collected.

Headspace samples were analysed in a gas chromatograph with a thermodesorption unit (GC) (6890 series, Agilent, Santa Clara, USA) connected to a mass spectrometer (MS) (5973 series, Agilent, Santa Clara, USA). Collected volatiles were desorbed from the Tenax in a thermodesorption trap unit (Gerstel, Mülheim, Germany) by heating from 25 °C to 250°C (5 min hold) at a rate of 60 °C min^{-1} in splitless mode. Released compounds were focused in a cold trap (ID 1.80 mm) filled with glass beads (diam. 0.75-1.00 mm) at a temperature of −50 °C. By flash heating of the cold trap to 250 °C at 12 °C sec^{-1}, volatiles were transferred to the analytical column (60 m x 0.25 mm ID, 0.25 µm film thickness, DB-5, J&W, Folsom, USA). Oven temperature programme started at 50 °C (1 min hold) and rose at a rate of 20 °C min^{-1} to 100 °C, subsequently increased at a rate of 4 °C min^{-1} to 280 °C (1.5 min hold) and finally rose up to 300 °C at a rate of 10 °C min^{-1}. Column effluent was ionized by electron impact ionization at 70 eV. Mass scanning was carried out from m/z 40 to 300 with

5.36 scans sec^{-1}. Compounds were identified by comparison of mass spectra with those of NIST, Wiley libraries and the Wageningen Mass Spectral Database of Natural Products. Identity was confirmed by comparison of retention index described in the literature (Adams 1995) and the respective index calculated during this study. Emission rates were quantified for compounds that were detected in at least 50% of the samples of one of the treatments.

Nectar Analyses

To examine the effect of herbivory on nectar secretion and sugar content, nectar was collected from the plants at 47 ± 1 h after start of the treatment. Around 9:00 am, one hour before collecting the nectar, the air humidity was increased to approximately 80% r.h. using a humidifier (Defensor 3001, Walter Meier Climate International ltd., Pfäffikon, Switzerland). To standardize for the stage of flowers, nectar was collected from the five distal flowers of five top flowering branches. The nectar was collected using 5 μL glass capillary tubes (Sigma Blaubrana intramark) with adjusted tips (Bruinsma *et al.* 2008) to determine nectar volume and approximately 10 μL nectar was used for further analysis: 10% for sugar and 90% for glucosinolate analysis. The obtained nectar per plant was stored in an Eppendorf tube at -20 °C until further analysis.

The sugar composition of the nectar was determined using high performance liquid chromatography (HPLC) analysis. The samples were diluted 10 times and injected in a Dionex BioLC system, equipped with a GS50 gradient pump, a CarboPac PA1 Analytical Column 4 x 250 mm with a CarboPac Guard Column 4 x 50 mm, and an ED50 electrochemical detector. The column was eluted with 100 mM NaOH at 1 mL min^{-1} and kept at 25 °C. Sugar concentrations were determined in g mL^{-1} using Chromeleon Software version 6.60 (Dionex Corporation, Sunnyvale, CA). Nectar samples of in total 22 infested and 21 control plants were collected.

For glucosinolate analysis the nectar was dissolved in 500 μL MeOH, desulphatased on a DEAE-Sephadex A25 column, and analysed by using HPLC as described previously (van Dam *et al.* 2004). Glucosinolate detection was performed with a photodiode array detector (200-350 nm) with 229 nm as the integration wavelength. For quantification, sinigrin (Sigma, St. Louis, MO, USA) was used as an external standard. To calculate the concentrations of glucosinolates, correction factors at 229 nm were used (Buchner 1987, European Commission 1990). Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV absorption spectra with standards provided by M. Reichelt, Max Planck Institute of Chemical Ecology (Jena, Germany) and certified rape seed standard (Community Bureau of Reference, code BCR-367R).

POLLINATOR RESPONSES TO HERBIVORE INFESTATION – GREENHOUSE EXPERIMENT

Pollinator preference was observed in two-choice experiments with one herbivore-infested and one control plant. The plants were positioned in a gauze tent (l × w × h: 293 cm × 200 cm × 230 cm) in a greenhouse compartment (22 ± 2 °C, 60 ± 10% r.h., L16:D8). For each experiment, new sets of plants were used and the position of the treatments was alternated. In total, 12 pairs of plants were tested in each of the experiments with the three pollinator species observed. Three pollinator species were studied in these experiments: adults of *P. rapae*, *A. mellifera* and *E. balteatus*. The larvae of *P. rapae* feed on *B. nigra* leaves and flowers, the adults only feed on flower nectar and can serve as pollinators. Only male butterflies were used in the experiments. Female butterflies showed a strong tendency to oviposit do not visit flowers often, not even when starved. Male butterflies are as efficient pollinators of *B. nigra* plants as female butterflies (D. Lucas-Barbosa, unpublished results). For *E. balteatus* and *P. rapae*, the number and duration of visits to flowers of each plant were recorded during 20 min. Eight individuals were released after being starved (fed only with water) since the previous evening. They were marked individually with fluorescent powder (Swada FIESTA daylight fluorescent colours series T, Stalybridge, UK) for *E. balteatus*, or with a number on the wings written with a permanent marker pen for *P. rapae*. Their behaviour was recorded with a handheld computer (Psion Workabout, Psion Techlogix Inc., Mississauga, Canada), programmed with The Observer software (version 4.1, Noldus Information Technology, Wageningen, NL). *Pieris rapae* experiments were performed in the morning; *E. balteatus* and *A. mellifera* were observed in the afternoon. For *A. mellifera*, the number of individuals visiting a plant during 9 minutes was recorded with a camera (Panasonic, NV-GS230EG/EF/EK, Panasonic Corporation, Kadoma, Osaka, Japan). The number of bees present on a plant was counted every 30 s. The mean duration of all bee visits was calculated based one randomly chosen minute of the 9 min recorded.

POLLINATOR RESPONSES TO HERBIVORE INFESTATION – FIELD EXPERIMENT

Plants that had received the same treatments as in the greenhouse experiments were transferred to a field, in the vicinity of Wageningen, and monitored on 13 days for the number and duration of visits by naturally occurring pollinators in June and July 2008. The field area used for the experiments was of about 10 m x 20 m, and it was surrounded by grass. Each plant was monitored for 10 min at the end of the morning (10 am and 12 am) and number and duration of visits by bees, syrphid flies, and other flower visitors were recorded using a handheld computer (Psion Workabout) programmed with The Observer software (version 4.1, Noldus Information Technology, Wageningen, The Netherlands). We observed pollinator visitation to 22 infested and 22 control plants in total.

STATISTICAL ANALYSIS

The greenhouse observation data were analysed using a paired t-test for the number of visits to each plant and general linear model (GLM) for the duration of the visits, with treatment and the replication of experiment as fixed factors. The data for the duration of the visits was ln-transformed for all three pollinator species. The data of the field experiment were also analysed using a paired t-test for the number of visits and GLM for the duration of the visits, with treatment and day as fixed factors. Nectar volume and sugar concentrations were not normally distributed and, therefore, analysed with a Mann-Whitney U test. Glucosinolate levels in the nectar were compared using a t-test. All analyses were performed with SPSS 15.0.

Projection to Latent Structures Discriminant Analysis (PLS-DA) (SIMCA P+ 12.0, Umetrics AB, Umeå, Sweden) was used to determine whether the volatile composition of flower and leaf samples from control and infested plants could be separated based on the composition of the volatile blend. To determine significant differences in emission of the compounds identified in the plant volatile blend we used ANOVA or Kruskal-Wallis tests when assumptions of normality were not met.

Results

PLANT VOLATILE EMISSION

Plants responded systemically to herbivore infestation by *P. brassicae* caterpillars: the volatile blend emitted by flowers changed in response to folivory (Table 1). A PLS-DA including volatiles emitted by flowers and leaves from caterpillar-infested and control plants resulted in a model with three principal components; the first 2 principal components explained 50 and 12% of the total variance, respectively. The second principal component to a large extent separated samples of flowers of control plants from samples of flowers of infested plants (Fig 1). Plants exposed to folivory emitted several compounds in larger amounts and others in smaller amounts from their flowers. For instance, the emission of acetophenone by flowers was increased after folivory, whereas the emission of benzaldehyde was lower than that from flowers of intact plants (Table 1). Emission of the monoterpene sabinene was also down-regulated in plants subjected to folivory, whereas the emissions of the monoterpene alcohol (*Z*)-pinocarveol and of the monoterpene aldehyde myrtenal were up-regulated. The emission of the homoterpene (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) was also increased in flowers in response to folivory. We did not detect changes in volatile emission by leaves in response to herbivore infestation (Table 1; Fig. 1). Volatile blends produced by flowers and leaves of *B. nigra* plants are quantitatively and qualitatively different. Total volatile emission per unit of biomass of *B. nigra* flowers is much higher when compared to the emission by leaves, irrespective of the treatment (Table 1). The monoterpene limonene was exclusively emitted by leaves, whereas other monoterpenoids, such as β-myrcene and sylvestrene, were characteristic of the flower blend.

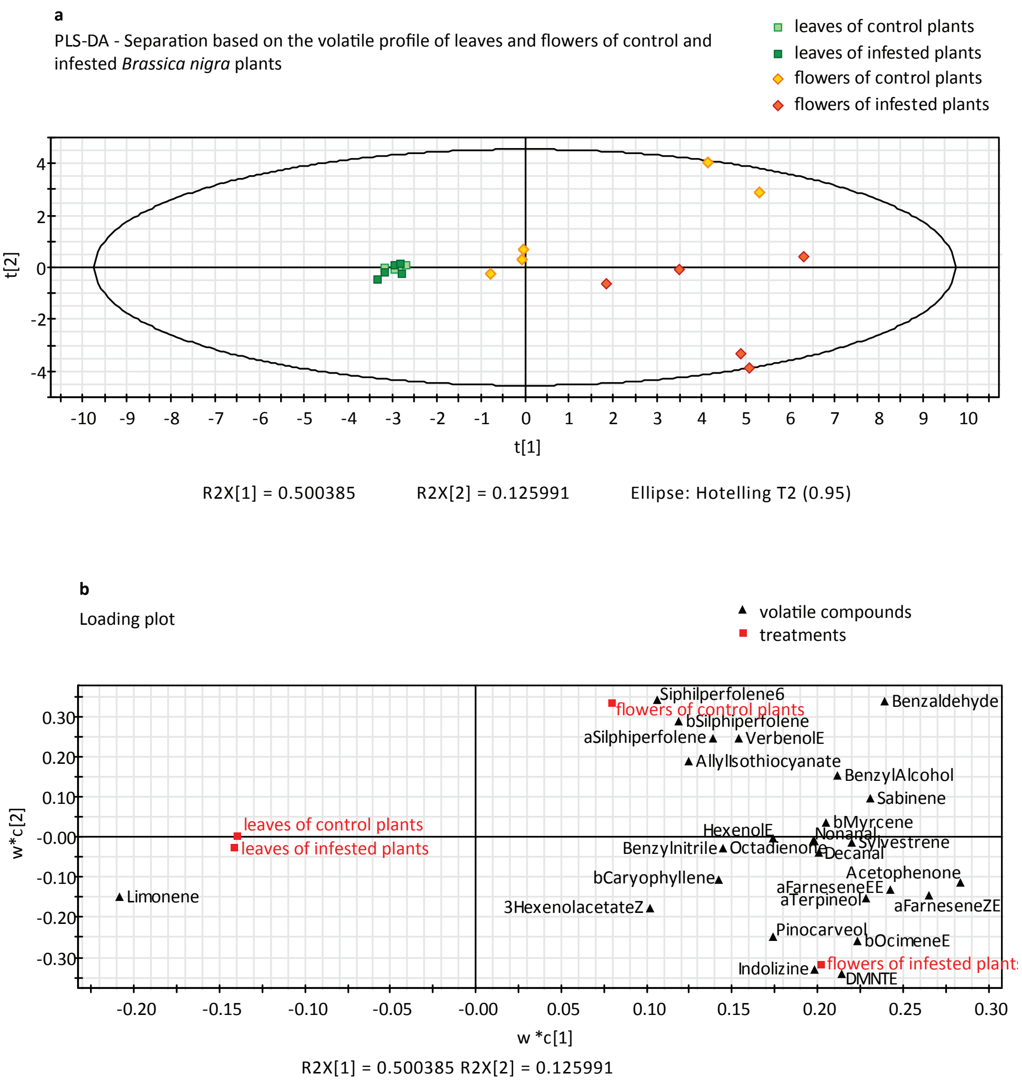

Fig. 1 Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile compounds collected from leaves and flowers of leaf-infested *Brassica nigra* plants, and from non-infested control plants. Plants were infested with 100 second instar larvae of *Pieris brassicae* larvae 48 h prior to volatile collection. PLS-DA on the peak area (log-transformed data) of volatile compounds from headspace of *B. nigra* plants. a) Grouping pattern of samples according to the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; b) Contribution of each of the volatile compounds to the first two principal components is shown in the loading plot of the PLS-DA components.

Table 1 Volatile compounds from leaves and flowers of leaf-infested *Brassica nigra* plants, and from non-infested control plants. Plants were infested with 100 second instar larvae of *Pieris brassicae,* 48 h prior to volatile collection.

	leaves of control plants	leaves of infested plants	flowers of control plants	flowers of infested plants
Volatile compounds	(Peak area/ plant biomass ± SD)*	(Peak area/ plant biomass ± SD)	(Peak area/ plant biomass ± SD)	(Peak area/ plant biomass ± SD)
Benzenoids				
Acetophenone	81 ± 41^{a}†	71 ± 67^{a}	369 ± 160^{b}	597 ± 121^{c}
Benzaldehyde	137 ± 82^{a}	158 ± 186^{a}	5455 ± 2662^{b}	4119 ± 1104^{c}
Benzyl alcohol	-‡	-	1448 ± 907^{b}	1214 ± 855^{ab}
Benzyl nitrile	-	-	42 ± 8^{b}	52 ± 31^{b}
Monoterpenoids				
Limonene	41 ± 19^{a}	55 ± 40^{a}	-	-
β-Myrcene	-	-	2314 ± 1055^{a}	2405 ±1332^{a}
Myrtenol	-	-	-	188 ± 57
β-Ocimene, (*E*)-	18 ± 19^{a}	19 ± 6^{a}	9357 ± 4521^{b}	13007 ± 8117^{c}
Pinocarveol, (*Z*)-	-	-	ss^{a}††	260 ± 118^{b}
1,7-Octadien-3-one, 2-methyl-6-methylene-	-	-	268 ± 134^{a}	210 ± 69^{a}
α-Terpineol	-	-	530 ± 300^{a}	489 ± 220^{a}
Sabinene	-	-	1334 ± 499^{a}	979 ± 335^{b}
Sylvestrene	-	-	3996 ± 1862^{a}	4838 ± 1760^{a}
Verbenol, (*E*)-	-	-	408 ± 167^{a}	464 ± 87^{a}
Homoterpenoids				
4,8-Dimethyl-1,3,7-nonatriene, (*E*)-	-	30 ± 8^{a}	174 ± 107^{a}	748 ± 539^{b}
Sesquiterpenoids				
β-Caryophyllene, (*E*)-	49 ± 27^{a}	-	170 ± 143^{a}	384 ± 378^{a}
α-Farnesene, (*E*,*E*)-	33 ± 18^{a}	28 ± 6^{a}	338 ± 107^{a}	526 ± 324^{b}
α-Farnesene, (*Z*,*E*)-	-	-	95 ± 30^{ab}	156 ± 68^{b}
7-(*α*)-H-Silphiperfol-5-ene	ss^{a}	32 ± 27^{ab}	151 ± 144^{ab}	111 ± 98^{b}
7-(*β*)-H-Silphiperfol-5-ene	ss^{a}	22 ± 18^{a}	475 ± 580^{b}	278 ± 324^{ab}
Silphiperfol-6-ene	-	-	120 ± 114^{b}	78 ± 62^{b}
Fatty acid derivatives				
Decanal	63 ± 35^{a}	57 ± 22^{a}	306 ± 198^{b}	447 ± 192^{b}
3-Hexen-1-ol, (*Z*)-	-	73 ± 65^{a}	251 ± 152^{b}	478 ± 247^{b}
3-Hexen-1-ol, acetate, (*Z*)-	ss^{a}	473 ± 348^{a}	844 ± 156^{a}	1944 ± 1178^{b}
Nonanal	35 ± 16^{a}	36 ± 12^{a}	286 ± 118^{b}	298 ± 91^{b}
Nitrogen containing				
Allyl isothiocyanate	ss^{a}	84 ± 18^{a}	904 ± 971^{b}	301 ± 167^{ab}
Indolizine	-	-	ss^{a}	109 ± 57^{b}
Total volatile emission	**577 ± 257**	**1139 ± 822**	**29880 ± 15094**	**34683 ± 17929**

*Values for peak area (mean ± SD) were divided by 10^5 and expressed per gram fresh weight. † different superscripts ($^{a, b, c}$) indicate significant differences between means at the 0.05 level (ANOVA or Kruskal-Wallis depending on data distribution). ‡ (-) indicates that compound was not detected in any sample. †† (ss) indicates that the compound was detected in a single sample.

COMPOSITION OF NECTAR OF CONTROL AND HERBIVORE-INFESTED PLANTS

The nectar volume collected from flowers did not differ between control and herbivore-induced plants (Mann-Whitney U: Z= -1.434, n = 24, *P* = 0.152; Table 2). Nectar from *B. nigra* contained two glucosinolates, sinigrin (2-propenylglucosinolate) and 4-hydroxyglucobrassicin (4-hydroxy-3-indolylmethylglucosinolate). The glucosinolate levels did not differ between nectar from control and herbivore-induced plants (t-test; sinigrin: t = -0.588, df = 39, *P* = 0.560; 4-hydroxyglucobrassicin: t = -1.670, df = 39, *P* = 0.103; Table 2). The nectar contained five sugars: mostly glucose and fructose, and small concentrations of sorbitol, sucrose and melezitose. The concentrations of all sugars were on average slightly higher in nectar from herbivore-induced plants than in nectar from control plants, and the total sugar concentration was 18% higher in nectar collected from herbivore infested-plants than nectar from control plants (Wilcoxon signed rank test, *P* = 0.043).

Table 2 Glucosinolate and sugar concentrations (mean ± SD) in nectar of control plants and leaf-infested *Brassica nigra* plants. Herbivore-infested plants were infested with 100 second instar larvae of *Pieris brassicae,* 48 h prior to nectar collection. Wilcoxon matched pairs test (α = 0.05; * $P \leq 0.05$) was used to determine difference in total sugar concentration.

		control plants	herbivore-infested plants
Nectar volume (µL)		1.0 ± 0.5	0.9 ± 0.5
	Sinigrin	271.0 ± 25.8	301.3 ± 43.8
	4-OH-GBC[1]	3.8 ± 0.3	4.5 ± 0.3
Glucosinolates (µM)			
total		**274.8 ± 26.1**	**305.8 ± 44.1**
	Sorbitol	5.6 ± 1.3	9.6 ± 1.3
	Glucose	1031 ± 206	1215 ± 550
	Fructose	1024 ± 216	1203 ± 479
	Sucrose	7.4 ± 4.5	9.5 ± 5.6
	Melezitose	7.7 ± 3.6	10.9 ± 6.9
Sugars (mM)			
total		**2077 ± 436**	**2449 ± 1056***

[1] 4-OH-GBC: 4-hydroxyglucobrassicin

POLLINATOR RESPONSES TO HERBIVORE INFESTATION - GREENHOUSE EXPERIMENT

Butterflies of *P. rapae* visited more flowers of control plants than of herbivore-infested plants (paired t-test: t_{11} = -3.647, *P* = 0.04; Fig. 2). Also the duration of the visits was longer on control plants than on herbivore-infested plants (GLM: treatment F_1 = 25.430, $P < 0.01$; Fig. 2). The effect of replication of the experiment and the interaction between replication and treatment were significant as well (F_{11} = 6.595, $P < 0.01$; treatment*replicate experiment F_{11} = 2.170, *P* = 0.014).

The average number of flower visits by *A. mellifera* did not differ between control and herbivore-infested plants (paired t-test: $t_9 = 0.110$, $P = 0.915$; Fig. 2). Visits to control plants were longer than to infested plants (GLM: treatment $F_1 = 32.035$, $P < 0.01$; Fig. 2). The differences between different replicate experiment and the interaction with the treatment were significant (replicate experiment: $F_9 = 20.529$, $P < 0.01$; treatment*replicate experiment $F_9 = 4.013$, $P < 0.01$).

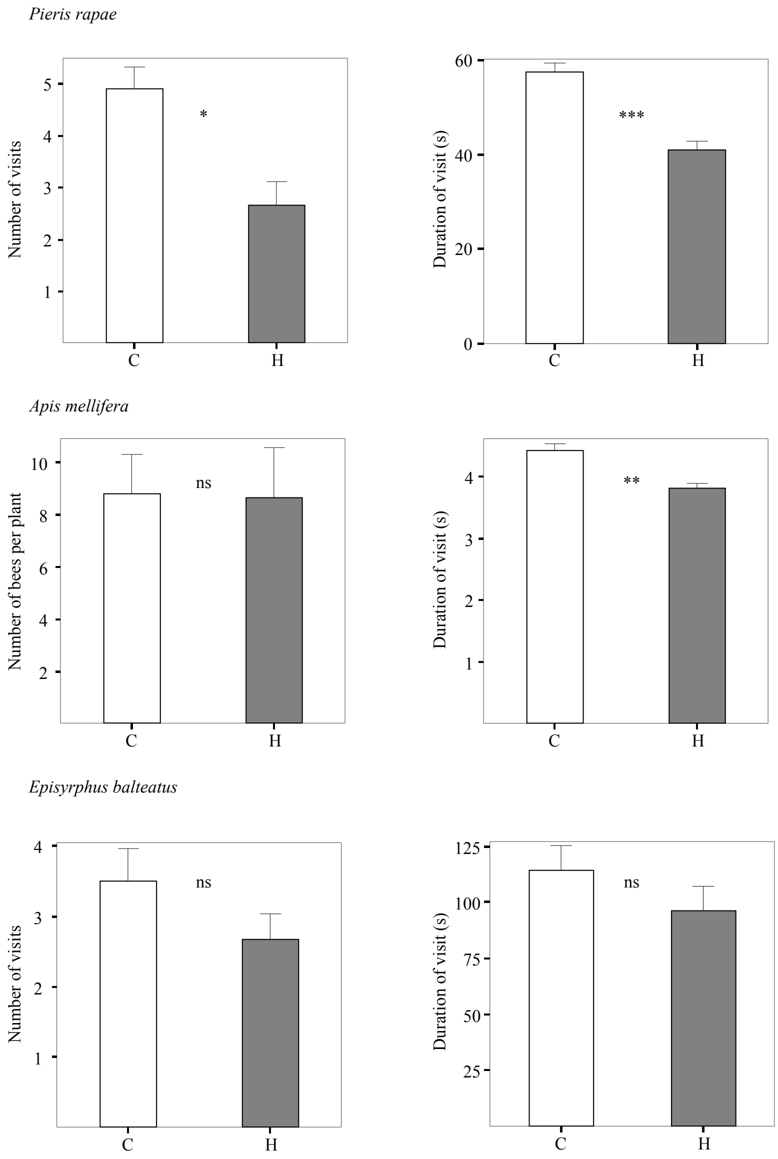

Fig. 2 Number and duration of flower visits (mean ± SE) on herbivore-infested (H) and non-infested control plants (C) in greenhouse experiments for *Pieris rapae* (N = 12), *Apis mellifera* (N = 10) and *Episyrphus balteatus* (N = 12); ns: $P > 0.05$, *: $P < 0.05$, ***: $P < 0.001$. In total, visitation to 12 pairs of plants was monitored, in each of the experiments with the three pollinator species observed.

The number of visits to herbivore-infested and control plants by the syrphid fly *E. balteatus* did not differ significantly between treatments (paired t-test: $t_{11} = 1.820$, $P = 0.096$; Figure 2). The duration of the visits also did not differ between control plants and herbivore-infested plants (GLM: treatment $F_1 = 2.985$, $P = 0.085$; Figure 2). The duration of visits did differ between replicate experiment ($F_{11} = 3.220$, $P < 0.01$) and the interaction between treatment and experiment replicate was significant (treatment*replicate experiment $F_{11} = 2.152$, $P = 0.016$).

POLLINATOR RESPONSES TO HERBIVORE INFESTATION - FIELD EXPERIMENT

In the field, flowers were visited mostly by syrphid flies, honeybees, solitary bees and bumble bees. Only syrphid flies and honeybees were visiting in sufficiently high numbers to allow statistical analysis. The average duration of flower visits by syrphid flies was longer on control plants than on herbivore-infested plants (GLM: treatment $F_1 = 8.432$, $P = 0.005$; day $F_{12} = 3.548$, $P < 0.01$; treatment*day $F_{12} = 1.419$, $P = 0.170$; Fig. 3). The number of syrphid flies visiting the flowers of control and herbivore-infested plants were not significantly different (GLM: treatment $F_1 = 0.791$, $P = 0.376$) nor were there any significant day or interactive effects (day $F_{12} = 13.493$, $P < 0.01$; treatment*day $F_{12} = 0.488$, $P = 0.918$; Fig. 3). The visits of honeybees to flowers of control or herbivore-infested plants did not differ in the number or duration (GLM number: treatment $F_1 = 0.176$, $P = 0.676$; day $F_{12} = 13.959$, $P < 0.01$; treatment*day $F_{12} = 0.754$, $P = 0.695$; GLM duration: treatment $F_1 = 0.854$, $P = 0.360$; day $F_{12} = 1.712$, $P = 0.103$; treatment*day $F_{12} = 2.984$, $P = 0.006$).

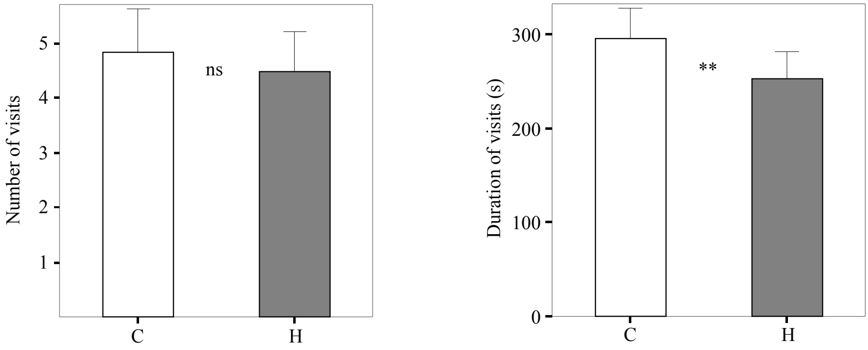

Fig. 3 Number and duration of flower visits of syrphid flies to undamaged (C) and herbivore-induced (H) *Brassica nigra* plants in the field (ns: $P > 0.05$, **: $P < 0.01$). In total, pollinator visitation to 22 infested and 22 control plants was monitored.

Discussion

Induced responses of plants to herbivory do not only affect herbivores and their natural enemies, but can also affect pollinator behaviour. Changes in plant odours or rewards offered by flowers can alter pollinator visitation, which in turn can affect pollination success, and subsequent fruit and seed set. Here, we show that herbivory by *P. brassicae* caterpillars on the leaves of black mustard plants affected pollinator behaviour both in the greenhouse and in the field. Plants responded to folivory with changes in floral volatile emission and increased sugar concentration in the nectar. Our results show that the plant-mediated effects of folivory on flower-pollinator interactions are dependent on pollinator species, similar to reports on the effects of florivory in *Centrosema virginianum* and *Eryngium yuccifolium* (Cardel & Koptur 2010, Danderson & Molano-Flores 2010). Different pollinators may exploit different plant cues, including long-range cues as well as contact cues, or the same cues in different ways. The general tendency of unchanged number of visits but shorter duration per visit to herbivore-infested plants could increase pollen transfer between flowers and enhance pollination effectiveness. Indeed, the correlation between pollen deposited on the flowers of the brassicaceous *Raphanus raphanistrum* and the duration of the visitation by butterflies and honeybees is weak (Conner & Neumeier 1995). Here, we found that nectar offered by infested plants contained more sugar than nectar offered by control plants. Flowers should offer nectar that is sweet enough to stimulate consumption and at the same time in small enough amounts to encourage pollinators to visit other flowers of the same plant species (Kessler & Baldwin 2007). Therefore, the sweeter nectar offered by infested *B. nigra* plants may increase outcrossing rates and herbivory-induced changes in flowering *B. nigra* plants do not seem to result in a trade-off with pollination. *Brassica nigra* plants are known to compensate for herbivory by *P. brassicae* (Blatt *et al.* 2008, Lucas-Barbosa *et al.* 2013), and results of the present study provide a step further in understanding how flowering *B. nigra* plants respond to herbivory and how compensation is achieved.

Emission of Herbivore-Induced Volatiles by Flowering Plants

Our results show that *B. nigra* responds to leaf herbivory with changes in volatile emission by the flowers. It is remarkable that volatile emission in response to folivory by *P. brassicae* caterpillars changed in flowers, but not in leaves. Plants in the vegetative stage typically increase leaf volatile emission in response to herbivore infestation (Mumm & Dicke 2010). Apparently, this can be quite different for plants in the flowering stage. In this context, it is worthy to note that in *Nicotiana attenuata* plants, the herbivore-dependent induction of the phytohormones ethylene, jasmonic acid and JA-isoleucine is attenuated in the leaves during the flowering stage in contrast to the non-flowering stage (Diezel *et al.* 2011). So far, only few studies have analysed floral volatile emission after herbivory and the results are variable. For example, in *Nicotiana suaveolens,* folivory did not change floral volatile emission (Effmert *et al.* 2008), whereas in *Solanum peruvianum* folivory induced volatile production, in both leaves and flowers, and the induction of a few compounds was tissue specific (Kessler & Halitschke 2009, Kessler *et al.* 2011). Plant-pollinator interactions were negatively affected by herbivory in these cases (Kessler & Halitschke 2009, Kessler *et al.* 2011). Flowering *Sinapis alba*, a brassicaceous plant, responded to damage by two species of phloem feeders by decreasing floral volatile emission. These changes did not disrupt the behaviour of the natural enemies of the aphids, when searching for host or prey (Pareja *et al.* 2012). This further underpins that investigating induced plant responses during the generative phase is seriously needed to understand how plants respond to herbivory when reproduction has started and how these changes may influence the behaviour of mutualistic insects such as pollinators.

Long-Range Cues and Pollinator Behaviour

Changes in floral volatile emission in response to folivory did not affect the attraction of syrphid flies and honeybees to the plants; herbivore-infested and control plants received similar numbers of visits by syrphid flies and honeybees. *Pieris rapae,* however, visited fewer infested plants than control plants. This could be due to their response to changes in floral odours. These butterflies can indeed detect herbivore-induced phytochemical changes from a distance. The olfactory neurons in the antennae of both sexes of *P. rapae* butterflies detect different classes of plant volatile compounds in a dose-dependent manner, including the fatty acid derivatives (*Z*)-3-hexen-1-ol and (*Z*)- 3-hexen-1-ol–acetate, the terpenoids myrcene and limonene, the benzenoid benzaldehyde and the glucosinolate hydrolysis products allylisothiocyanate and benzyl nitrile (*syn.* 2-phenylacetonitrile) (Topazzini *et al.* 1990, van Loon *et al.* 1992, Omura *et al.* 1999). Detection of changes in quantities or ratios of the compounds emitted by flowers in response to folivory offers a plausible sensory basis for the behavioural preference for flowers of control plants that we observed for males butterflies of *P. rapae*. Female butterflies and moths can also use herbivore-induced plant volatiles when searching for oviposition sites. Changes in the plant volatile blend is known to render flowers attractive for visitation, but do not necessarily render the plant more attractive for oviposition by adult herbivores (Reisenman *et al.* 2010). Therefore, herbivore-induced responses by flowering plants can influence feeding and oviposition behaviour of adult female herbivores differently (Reisenman *et al.* 2010). We expect that changes in flower odours might be associated by a hungry adult herbivore with the quality of the reward offered by the flowers. In this study, male *P. rapae* butterflies were more attracted to control plants, although they benefited from higher sugar content present in flowers of herbivore-infested plants, and probably saved energy by spending less time on each flower when feeding on infested plants. Thus, this preference seems to be non-adaptive for male butterflies at this point in time, and we expect that selection acts on butterflies to associate odour cues with nectar quality.

Contact Cues and Pollinator Behaviour

Secondary metabolites and sugar concentration in nectar might influence the duration of the visitation by pollinators. Glucosinolates are typical defensive plant secondary metabolites of brassicaceous plants (Hopkins *et al.* 2009). The presence of defensive compounds in nectar might be counterintuitive, because nectar is meant to reward pollinating insects. Defensive compounds may, however, play a role in maximizing outcrossing rates (Klinkhamer & Dejong 1993, Adler 2000, Kessler & Baldwin 2007, Kohler *et al.* 2012) and enhance plant reproduction. Secondary metabolites in nectar could decrease the duration of a flower visit by a pollinator and, consequently, decrease nectar consumption, while still transporting pollen between flowers (Kessler & Baldwin 2007). Our results show that glucosinolates are constitutively present in the nectar of *B. nigra* plants. To our knowledge this is the first study that shows that glucosinolates are present in nectar of brassicaceous plants. Sinigrin was the main glucosinolate present in *B. nigra* nectar and 4-hydroxy-glucobrassicin was present in smaller quantities. Sinigrin is also the main glucosinolate in *B. nigra* leaves and flowers (Smallegange *et al.* 2007, Gols *et al.* 2008). Folivory was shown to increase glucosinolate concentration in leaves, but not in flowers (Smallegange *et al.* 2007) or in nectar (this study). Butterflies and honeybees spent less time on herbivore-infested plants than on control plants. The reduced duration of flower visits by the butterflies and honeybees that we observed is likely influenced by higher sugar concentration in nectar. The behaviour of the syrphid fly *E. balteatus* was not influenced by folivory. Indeed, *E. balteatus* was observed to collect mainly pollen from *B. nigra* flowers (C. J. M. ten Broeke and D. Lucas-Barbosa, personal observation). Whether plants respond to herbivore infestation with changes in pollen quantity and quality remains to be investigated.

Herbivore-Induced Plant cues and Plant Fitness

To increase chances of reproduction, plants may enhance interactions with pollinators (Strauss & Murch 2004). In this study, the general tendency for shorter visit duration to herbivore-infested plants could enhance pollination effectiveness, and subsequently plant fitness. Indeed, *B. nigra* plants infested with *P. brassicae* produced as many seeds as non-infested plants and compensated for herbivory in terms of seed production (Lucas-Barbosa *et al.* 2013). This might be particularly important when herbivores may become a threat to the flowers. Second instar larvae of *P. brassicae* migrate from the leaves to the flowers of *B. nigra* and start feeding there (Smallegange *et al.* 2007, Lucas-Barbosa *et al.* 2013). A shift from folivory to florivory can directly influence pollination success by decreasing the number of flowers, increasing flower asymmetry, decreasing petal size and floral conspicuousness (Galen 1999, Krupnick & Weis 1999, Cardel & Koptur 2010, Irwin & Brody 2011). *Brassica nigra* can anticipate future damage and maximize its chances of reproduction in different ways. For instance, *B. nigra* can accelerate seed production in response to the deposition of *P. brassicae* eggs; consequently, the plant produces seeds before the flowers are consumed by the caterpillars (Lucas-Barbosa *et al.* 2013). Because the seeds are not eaten by the caterpillars, the plant can safeguard its reproductive output in this way, and have compensated for herbivory in terms of seed production (Lucas-Barbosa *et al.* 2013). Our results suggest that *B. nigra* can also maximize its interactions with pollinators in response to herbivore attack. Here, folivory reduced the duration of flower visits by pollinators without affecting the number of flower visits. It is not straightforward, however, to translate effects of herbivory on pollinator visits into plant fitness because of the complexity of herbivore-plant-pollinator interactions, particularly in such a generalized system. *Brassica nigra* plants are, in nature, visited by a large number of bees and syrphid flies species. Additionally, whether pollinator visits can be translated into efficient transfer of pollen, and subsequently plant fitness, is probably partially influenced by self-fertilisation and/or stigmatic clogging that may prevent seed set (Strauss *et al.* 2004). Just like interactions of plants with herbivores may influence pollinator behaviour, also pollinator activities may affect herbivore behaviour, for example, by reducing larval feeding activity (Tautz & Rostas 2008). Moreover, to assess the ecological consequences of herbivory-induced plant responses for plant fitness, effects of herbivory on pollinator behaviour still need to be integrated with studies that also investigate effects of flowering on the behaviour of other plant mutualists, such as predators and parasitoids (Dicke & Baldwin 2010; Lucas-Barbosa *et al.* 2011).

Conclusions

In this study, a trade-off between plant defence and pollination was not evident, in the sense that the number of visits by pollinators was not reduced, but the duration of visits was affected. This could be beneficial for the plant and increase pollen transfer between flowers while reducing nectar consumption by the pollinating insects. Indeed, herbivory does not lead to a reduction in the number of seeds produced by *B. nigra* plants (Lucas-Barbosa *et al.* 2013). Our results increase the current understanding of how plants in the flowering stage respond to herbivore infestation and can eventually compensate for herbivory. Here, plants responded to folivory with changes in nectar sugar concentration and in floral odour emission. Evidence is growing that not only florivory, but also folivory can influence induced responses in flowers and, consequently, pollinator behaviour. Yet, to translate effects of herbivore-induced plant responses to plant fitness, these results should be placed in a community context; a better understanding is required of how induced responses by plants in the flowering stage affects the behaviour of mutualistic insects such as pollinators, but also that of predators and parasitoids.

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Chapter 5

Changing colours and odours: how plant responses to pollination and herbivory affect the behaviour of flower visitors

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Abstract

Plants evolved strategies to attract pollinators that are essential for reproduction. However, plant defence against herbivores may trade-off with pollinator attraction. Here, we have investigated the role of inducible plant secondary metabolites in such a trade-off. Our objective was to reveal the mechanisms underlying the effects of induced plant responses to herbivory and pollination on the behaviour of flower visitors. Thus, we assessed how responses of *Brassica nigra* plants to pollination and insect herbivory affect the behaviour of flower visitors when caterpillars were feeding on the leaves or on the flowers of *B. nigra* plants. Subsequently, we investigated how these plants respond to pollination and insect herbivory in terms of the production of volatile and non-volatile compounds, and whether plant responses to herbivory interfere with responses to pollination. Our results show that butterflies use different cues when searching for an oviposition site or a nectar source. Syrphid flies visited preferably recently opened flowers, and previous pollination did not influence their behaviour. Plants respond to pollination and herbivory with changes in the profile of volatiles and non-volatiles of *B. nigra* flowers, which can influence the colour-odour association exploited by pollinators. Systemic responses to herbivores can interfere with local responses to pollination that are essential in optimizing plant reproductive success. We discuss the results in the context of the trade-off between plant defence and pollinator attraction and conclude that both herbivores and pollinators induced important phenotypic changes in flowers. Therefore, these responses must be addressed in an integrated way because in nature plants are exposed to herbivores and pollinators at the same time.

Keywords: flowers, florivory, folivory, herbivore-induced plant volatiles, phenolics, pollination.

Introduction

Plants have evolved strategies to attract pollinators that are essential for reproductive success as well as resistance and compensation mechanisms against herbivore damage to reduce or prevent fitness loss (Fornoni 2011, Lucas-Barbosa *et al.* 2013, Quintero & Bowers 2013, Schiestl & Johnson 2013). Induced plant defences to herbivores may trade-off with plant reproduction that is mediated by pollinators (Kessler & Halitschke 2009, Dicke & Baldwin 2010, Lucas-Barbosa *et al.* 2011). Yet, research in the fields of the ecology of plant-pollinator interactions and plant defence to herbivores has remained separated although pollinators and herbivores can both influence flower traits and impact plant fitness (Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011).

The diversity of flowers in angiosperms is one of the most remarkable features found in the plant kingdom (Schoonhoven *et al.* 2005, Harder & Barrett 2006). Flowers are the result of selection on traits to optimize plant reproductive success (Harder & Barrett 2006, Schiestl & Johnson 2013). The evolution of flowers has largely been associated with the behaviour of pollinators that are rewarded with nectar and pollen when they help plants to reproduce. About two thirds of all angiosperm plants depend on insects for pollination. Indeed, pollinator-mediated selection on flower traits can be strong, and the molecular basis of the diversity achieved in flower colours and odours is simply fascinating (Schiestl & Johnson 2013). To lure pollinators, flowering plants emit complex odour bouquets and display a wide spectrum of colours in flowers (Raguso 2008). To maximize reproduction, plants can respond to pollination with changes in flower colours and odours and in this way guide pollinators to flowers that have not been pollinated yet (Weiss 1991, Rodriguez-Saona *et al.* 2011). Most pollinator species orient themselves by exploiting visual and odour cues simultaneously, and colours and odours of flowers can be equally important in determining pollinator preferences (Burger *et al.* 2010, Milet-Pinheiro *et al.* 2012). This suggests that pollinators might exploit specific colour-odour combinations that may result from downstream regulation of a common precursor (Knudsen & Gershenzon 2006, Glover 2011, Dormont *et al.* 2014). For instance, aromatic volatiles and flavonoid compounds are produced through the phenylpropanoid pathway. Flavonoids are the most common flower pigments in nature. Aromatic volatile compounds such as benzenoids have been widely associated with pollinator attraction (Knudsen & Gershenzon, 2006). Changes in the biosynthesis of one of these groups of compounds can lead to changes in the production of the others and modify the colour-odour combination in flowers (Dormont *et al.* 2014). Specificity in colour-odour combinations may be influenced by phenotypic changes induced upon pollinator visitation (Dormont *et al.* 2014).

Besides pollinators, insect herbivores can also induce phenotypic changes in flower traits and impact plant fitness (Herms & Mattson 1992, Kessler & Halitschke 2009, Dicke & Baldwin 2010, Lucas-Barbosa *et al.* 2011). Herbivores can impact plant fitness directly by consuming flowers and also indirectly through induced phytochemical changes that can result in shifts in biosynthetic pathways and alter interactions between other antagonists and plant mutualists

(Kessler *et al.*, 2011). Induced plant defence mechanisms against herbivorous insects have evolved to reduce damage or compensate for damage (Dicke & Hilker 2003, Agrawal 2011, Fornoni 2011). This may alter the quality of nectar and pollen, and, thus, influence feeding preferences of flower visitors. In fact, in most studies performed so far, herbivore damage to leaves and flowers negatively affected pollinator behaviour (Kessler & Halitschke 2009). When the behaviour of beneficial insects is negatively affected, a potential conflict between induced plant responses and plant reproduction may lead to negative consequences for plant fitness (Kessler & Halitschke 2009, Majetic *et al.* 2009, Lucas-Barbosa *et al.* 2011).

Plant responses to the activities of pollinators and herbivores can involve the same classes of secondary compounds (Dudareva *et al.* 2004, Brodmann *et al.* 2008, Wiemer *et al.* 2009, Schiestl 2010). Yet, to our knowledge, no studies have investigated whether and how herbivore-induced plant responses can affect responses to pollination and lead to changes in pollinator behaviour even though, in nature, plants are exposed to pollinators and herbivores simultaneously. Thus, in this study we investigated the mechanisms underlying the effects of induced plant responses to herbivory and pollination on the behaviour of flower visitors. More specifically, our objectives were to investigate: a) how *Brassica nigra* plants respond to pollination and insect herbivory by *Pieris brassicae* caterpillars in terms of volatile and phenolic compounds; b) whether plant responses to herbivory interfere with responses to pollination and; c) how these plant responses affect the behaviour of insect pollinators when caterpillars were feeding on the leaves or on the flowers of *B. nigra* plants.

Materials and methods

Study system

Pieris brassicae L. (Lepidoptera: Pieridae) is a gregarious species and female butterflies lay batches of up to 100 eggs on the underside of leaves. When eggs hatch, caterpillars initially feed gregariously on leaves of a flowering *Brassica nigra* Koch (Brassicaceae) plant, but second-instar larvae soon move to flowers. This lepidopteran is a specialist herbivore of plants in the family Brassicaceae and can cope with the main defensive compounds these plants produce. *Pieris* butterflies can detoxify glucosinolates and sequester phenolic compounds from brassicaceous plants (Wittstock *et al.* 2004, Ferreres *et al.* 2009, Winde & Wittstock 2011). *Episyrphus balteatus* (De Geer) (Diptera: Syrphidae) is one of the most common syrphid fly species worldwide (Jauker & Wolters 2008). Larvae of syrphid flies feed on aphids, and therefore syrphid flies have been widely used as biological control agents. Adult of *E. balteatus* feed on nectar and pollen, but collect mainly pollen from *B. nigra* flowers. *Episyrphus balteatus* adults serve as pollinators, increasing the fitness of *Brassica* plants (Jauker & Wolters 2008). *Brassica nigra* is an annual plant that is considered to be obligately outcrossing and in nature it is pollinated by various insects including bees, syrphid flies and butterflies (Conner & Neumeier 1995, Lucas-Barbosa *et al.* 2013).

INSECTS AND PLANTS

Pieris brassicae used in the experiments were obtained from a laboratory colony reared on Brussels sprouts (*Brassica oleracea* var. *gemmifera*) plants in a climate room (22 ± 1 °C, 50-70% r.h., L16:D8). The adults were provided with a 10% sucrose solution as food. *Episyrphus balteatus* pupae were obtained from Koppert B.V., Berkel en Rodenrijs, The Netherlands. In the cage where adults were kept (22 ± 1 °C, 50-70% r.h., L16:D8) a Brussels sprouts plant infested with aphids was present, known to promote the development of the female reproductive system (M. Kos, personal communication). Adult syrphid flies had access to sugar, pollen and water.

Seeds of an early-flowering accession (CGN06619) of *B. nigra* were obtained from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands) and multiplied by exposing plants to open pollination in the surroundings of Wageningen. Seeds collected from 25 plants were mixed to obtain seed batches to grow the experimental plants. For the greenhouse experiments, potted *B. nigra* plants were reared in a greenhouse compartment (23 ± 2°C, 50-70% r.h., L16:D8). Plants in the flowering stage 3.1, based on the classification for *Brassica napus* (Harper & Berkenkamp 1975), were used for the experiments.

PLANT TREATMENTS

Herbivore infestation treatment

Flowering plants were infested with one egg clutch of *P. brassicae* by exposing plants to butterflies in an oviposition cage (100 cm × 70 cm × 82 cm). While the plant was inside the oviposition cage, flowers were covered with a mesh bag to prevent being visited by the butterflies. The number of eggs on a plant was reduced to 30 by gently removing surplus eggs shortly after the plants had been removed from the oviposition cage. For the 120 h herbivore treatment, 50% of the caterpillars were removed from the plants 48 h after hatching to simulate predation and dispersal. At each time point, for every plant subjected to treatment, there was another untreated plant in the same growth stage that was used as control.

Pollination treatment

Flowers of *B. nigra* plants were hand-pollinated five days after the first flower had opened, with pollen from a different plant individual (cross-pollination). Plants in the same stage as treated plants not exposed to pollination were kept as control plants. For the 24 h treatment, all open flowers of the plants used for the pollination treatment were marked with a black thread after being hand-pollinated with a paint brush, so that pollinated flowers could be distinguished from unpollinated flowers before the experiments. As a control for the hand-pollination experiment, plants were also exposed to pollination by male butterflies. To ensure cross-pollination by butterflies, plants were put in pairs into an insect rearing tent

(75 cm × 75 cm × 115 cm) inside a greenhouse compartment (23 ± 2 °C, 70% r.h., L16:D8), one tent for control plants and the other to apply the treatment. Two male butterflies, 3 to 7 days after eclosion, were released into the treatment tent for 6 h (from 9:00 a.m. until 3:00 p.m.). In order to increase frequency of flower visitation and, consequently, efficiency of pollination, butterflies were starved for 16 h before the experiment.

Pollination and herbivore infestation treatment

The combined effect of pollination and herbivore infestation on induced changes in *B. nigra* plants was also quantified. Plants were infested with one clutch of 30 eggs as described above. On the day the caterpillars hatched, all open flowers of infested plants were hand-pollinated with pollen from a different plant. Plants of the same stage not exposed to either infestation or pollination were used as control plants. For the 24-h treatment, all pollinated flowers were marked with a black thread as describe above.

Effects of herbivory on host plant selection by butterflies

To test whether herbivore infestation influences host-plant selection by *P. brassicae* female butterflies, a choice between infested and non-infested *B. nigra* plants was offered to mated female butterflies, 24 h after caterpillars had hatched from eggs on infested plants. Experiments were carried out in a flight chamber set up (gauze tent of 293 cm × 200 cm × 230 cm in height), in a greenhouse compartment (25 ± 2 °C, 50-70% r.h.). The preference of sucrose-fed (10%, *ad libitum*) female butterflies was tested by scoring first landing and oviposition preference. A single butterfly was released at a time, at a distance of 80 cm from the plants. When a butterfly did not make a choice within 15 minutes, it was recorded under 'no response' and excluded from statistical analysis. On each experimental day, up to 15 butterflies were tested; we observed the behaviour of, in total, 50 adult butterflies on 5 days. On each day, different groups of plants were offered to the butterflies. Butterflies at 5 to 7 days after eclosion and two days after mating were used in the experiments. They were considered to be naïve because they had not been exposed to a plant as an adult prior to the bioassays. Each individual butterfly was used only once. The positions of the two groups of plants were interchanged after every 3 butterflies tested to compensate for unforeseen positional bias. Data were analysed using a general linear model (GLM) with the plant-pair included as a factor.

Effects of herbivory and pollination on the flower feeding preference of syrphid flies and butterflies

To test whether pollination and herbivore infestation affect the feeding preference of the syrphid fly *E. balteatus* and of *P. brassicae* butterflies, we observed the preference of adult insects for treated or control plants in two-choice situations. The control plant was always a plant that had neither been infested nor pollinated. There were 4 treatments: 1) leaf-infested plant (1 day after the caterpillars had hatched from the eggs); 2) flower-infested plant (5 days after the caterpillars had hatched from the eggs); 3) pollinated plant (1 day after plants had been pollinated); 4) the combined effect of pollination and leaf-infestation. Experiments were carried out in a flight chamber set up (gauze tent of 293 cm × 200 cm × 230 cm), in a greenhouse compartment (25 ± 2 °C, 50-70% r.h.). A single adult butterfly or syrphid fly was released at a time, at 80 cm from the plants. The first choice of an adult male or female insect to a treated or control plant was recorded. In this case, first choice was defined as the first plant the insect had contact with, either with a leaf or flower. We also recorded the duration of flower visitation and number of flowers visited on treated and control plants, using a handheld computer (Psion Workabout Pro), programmed with 'The Observer' (version 10, Noldus Information Technology, Wageningen, The Netherlands). Each observation lasted 15 min and when the insect did not make a choice within 5 min it was recorded under 'no response' and excluded from statistical analysis. Each individual adult insect was used only once. Syphid flies were starved for 4-8 h prior to the experiments. Adult butterflies were starved for 15-18 h before the start of the behavioural assays and mated females had the opportunity to lay eggs on Brussels sprouts plants, before being used in the experiment, because we had previously observed that female butterflies prioritize oviposition over feeding (D. Lucas-Barbosa, personal observation). Adult insects were used for the experiments within 7 days after eclosion and two days after copulation, in the case of mated butterflies. On each experimental day, up to 15 insects were tested; we observed the behaviour of at least 30 insects of each sex with 5-8 pairs of plants. Data were analysed using GLM with plant-pair included as a factor or with Wilcoxon signed-rank tests with the plant-pair as the experimental unit.

HEADSPACE COLLECTION OF PLANT VOLATILES AND ANALYSIS BY GC-MS

To investigate whether flowering *B. nigra* plants respond in terms of released volatiles to herbivore infestation and pollination, plant volatiles from the aerial parts of: 1) control plants, 2) infested plants, 3) pollinated plants, and 4) plants exposed to the combined effects of pollination and leaf-infestation (24 h after caterpillars hatched from eggs) were collected. Plant headspace volatiles were collected by enclosing aerial parts of plants of the different treatments in an oven bag (Toppits® Brat-Schlauch, polyester; 32 cm × 32 cm × 70 cm; Toppits, Minden, Germany) for 1.5 h. Headspace samples were then analysed in a gas chromatograph with a thermodesorption unit (GC) (6890 series, Agilent, Santa Clara, USA) connected to a mass spectrometer (MS) (5973 series, Agilent, Santa Clara, USA). We followed the methods outlined by Bruinsma *et al.* (2014) to collect, analyse, identify and quantify volatiles emitted by *B. nigra* plants subjected to the different treatments. Experiments were performed in a greenhouse compartment (22 ± 2 °C, 60 ± 10% r.h. L16:D8). We collected plant volatiles from the headspace of at least 5 plants per treatment.

Projection to Latent Structures Discriminant Analysis (PLS-DA) (SIMCA P+ 12.0, Umetrics AB, Umeå, Sweden) was used to determine whether the samples subjected to the different treatments could be separated based on the composition of the volatile blend. To determine significant differences between emission of volatile compounds by treated and non-treated plants, we used ANOVA or Kruskal-Wallis tests when assumptions of normality were not met.

Extraction of non-volatiles and analysis by RP-UHPLC-DAD-MS

To investigate whether pollination and feeding by *P. brassicae* caterpillars affect the UHPLC profiles of crude aqueous methanolic extracts of *B. nigra*, leaf and flower, both tissues of these plants were analysed and quantified. One and five days (24 h and 120 h) after the caterpillars had hatched from the eggs, leaves and flowers of each plant were harvested, frozen in liquid nitrogen and freeze dried. All herbivores were removed from the plants before freezing the samples. Dried samples were ground, weighed and stored at −20 °C. To analyse their non-volatile content, ground leaves and flowers of *B. nigra* subjected to different treatments were extracted with MeOH:H_2O (8:2). A total of 200 mg of each sample was extracted with 20 mL of methanol (HPLC grade, J.T.Baker®, Avantor Performance Materials, USA) - ultrapure water (Milli-Q, Merck Millipore, Billerica, USA) (8:2) by sonication at room temperature for 10 min (Gaspar *et al.* 2009). The solid-liquid mixture stood for 25 min after which the supernatant was filtered into a 20 mL glass vial using a disposable polypropylene syringe through a PTFE syringe filter (13 and 17 mm ф 0.45 μm, Grace, Deerfield, USA). Extracts were stored at −20 °C until analysis. At least 6 plants were used for each treatment and time point.

Resulting extracts were analysed using a reversed-phase UHPLC Agilent Eclipse XDB-C18 column (2.1 mm × 150 mm, 1.8 μm) equipped with a binary pump, a diode array detector (DAD), an autosampler and a thermostatic column compartment (all Agilent 1290 Infinity LC, Agilent Technologies, Santa Clara, USA). Two solvents were used as mobile phase: (A) pH = 3 buffer and (B) acetonitrile (HPLC grade, Sigma-Aldrich, St. Louis, USA). The pH = 3 buffer (200 mM) used as solvent A consisted of formic acid (160 mM, 99% pure, Thermo Fisher Scientific,Geel, Belgium), ammonium formate (40 mM, HPLC grade, Sigma-Aldrich, St. Louis, USA) and EDTA (0.04 mM, 98%, Sigma-Aldrich, St. Louis, USA) in ultrapure water (Villela *et al.* 2011). The following gradient was used: 7% B (t = 0), 18% B (t = 10 min), 65% B (t = 13.6 min), 7% B (t = 14 min), 7% B (t = 20 min). The flow rate was 0.45 mL min^{-1} and the injection volume was 2 μL. The column was kept at a temperature of 50 ˚C. During the entire UHPLC run DAD spectra were recorded from 200 to 800 nm, and for quantification of individual compounds, chromatograms were plotted and integrated at 330 nm. Projection to Latent Structures Discriminant Analysis (PLS-DA) (SIMCA P+ 12.0, Umetrics AB, Umeå, Sweden) was used to determine whether the samples subjected to the different treatments could be separated on the basis of their 330 nm profile. To determine significant differences between each of the quantified compounds in the aqueous methanolic extracts we used ANOVA or Kruskal-Wallis tests when assumptions of normality were not met.

Characterization of non-volatiles compounds

In this study, non-volatile compounds were characterized by their absorbance profile in the UV-Vis. Liquid chromatography-mass spectrometry (LC-MS) was used to determine the accurate molecular weight of some of the unknown compounds obtained from the sample separation by UHPLC. One sample of each treatment was analysed. Separated phenolic individual compounds were analysed by an Exactive mass spectrometer (Exactive MS) (Thermo Fisher Scientific, Waltham, USA), equipped with an electrospray ionisation (ESI) interface. Mass spectra were recorded in the negative and positive ionisation modes. The full scan mass range was set from m/z 100 to 1000, and at ultra-high resolution (100,000 at 1 Hz).

UV-Vis absorbance measurements of aqueous methanolic extracts

Absorbance spectra of the leaf- and flower extracts of plants subjected to different treatments were recorded to correlate changes in absorbance among treatments with changes in colour. Six samples of each treatment were scanned from 200 to 800 nm in 1 min, using a Cary® 100 UV-Vis spectrophotometer (Varian, Agilent Technologies, Santa Clara, USA) and quartz cuvettes of 3 mL. Prior to measurements, extracts were sonicated for 2 – 5 min and diluted (1:15) with MeOH:H_2O (8:2).

Results

Effects of herbivory on host plant selection by butterflies

When given a choice between leaf-infested and non-infested plants, mated female *P. brassicae* butterflies avoided to deposit eggs on plants infested with conspecific caterpillars (Fig. 1, GLM, $P = 0.003$). The butterflies also tended to land first on a non-infested plant when compared with a leaf-infested plant (Fig. 1, GLM, $P = 0.066$).

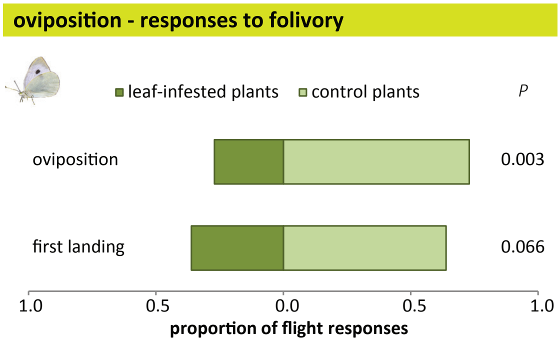

Fig. 1 Proportion of mated female *Pieris brassicae* butterflies that landed or oviposited first on infested and on non-infested *Brassica nigra* plants. Infested plants carried *P. brassicae* caterpillars that had hatched from the eggs 24 h before. Pairwise comparison of treatments with generalized linear model with binomial distribution. Four pairs of plants were used in these experiments and 40 butterflies responded to the test.

EFFECTS OF HERBIVORY AND POLLINATION ON THE FEEDING PREFERENCE OF SYRPHID FLIES AND BUTTERFLIES

Responses to herbivore infestation

We tested whether herbivory influenced the flower-feeding preference of syrphid flies and butterflies. We recorded the proportion of flight responses, the residence time and number of flowers visited on infested and non-infested plants, at two different time points, *i.e.* when caterpillars were feeding on leaves or when caterpillars were feeding on flowers. Male and female syrphid flies visited as many flowers of leaf-infested plants as flowers of non-infested plants (Fig. 2b, Wilcoxon signed rank test, *P* = 0.435), and time spent on each group of plants was similar (Wilcoxon signed rank test, *P* = 0.905). In contrast, florivory did influence behaviour of these pollinators. Female syrphid flies visited more flowers of non-infested plants (see supporting information in Appendix C, Fig. C1, Wilcoxon signed rank test, *P* = 0.004) and spent more time on these than on flowers of flower-infested plants (Wilcoxon signed rank test, *P* = 0.004), and discriminated between caterpillar-infested and non-infested plants from a distance (Fig. C1, GLM, *P* = 0.004), whereas the preference of male syrphid flies

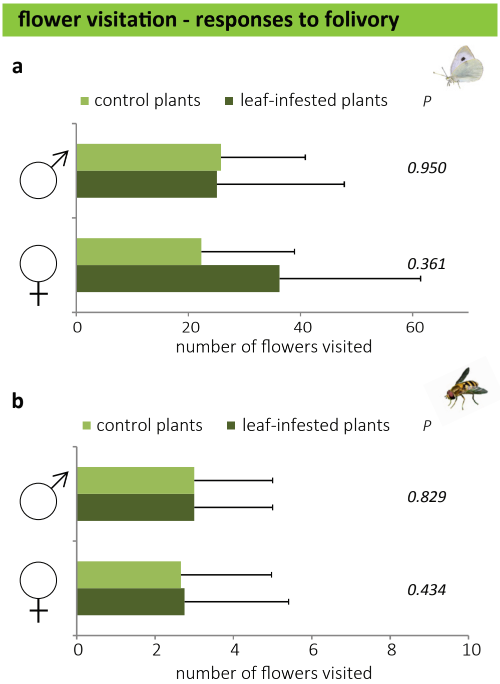

Fig. 2 Number of *Brassica nigra* flowers visited (mean + SD) by (a) male and female butterflies of *Pieris brassicae* and (b) syrphid flies of *Episyrphus balteatus* to leaf-infested and non-infested control plants. Infested plants carried *P. brassicae* caterpillars had hatched from the eggs 24 h before. Pairwise comparison of treatments with generalized linear model with Poisson distribution. At least 30 insects of each sex were tested with 5-8 pairs of plants.

was not significantly influenced by herbivory. Neither folivory (Fig. 2a) nor florivory (Fig. C1) influenced feeding preferences of male or female *P. brassicae* butterflies foraging for nectar sources on caterpillar-infested *B. nigra* plants when compared with non-infested plants.

Responses to pollination

We tested whether pollination influenced flower-feeding preference of syrphid flies and butterflies. Male and female butterflies rarely visited pollinated flowers (Fig. 3), and when they did, they spent less time on them than on an unpollinated flower (Wilcoxon signed-rank test, $P = 0.033$). In contrast, pollination did not influence the behaviour of syrphid flies. However, male syrphid flies visited more new flowers than old flowers of pollinated and unpollinated plants (Fig. 3).

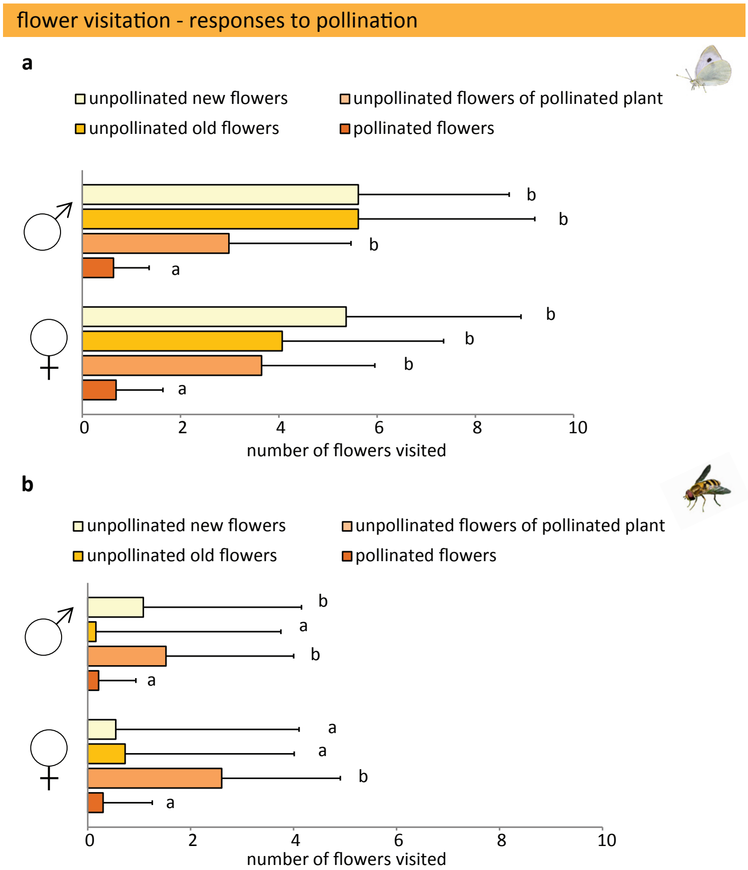

Fig. 3 Number of *Brassica nigra* flowers visited (mean + SD) by male and female butterflies of *Pieris brassicae* (a) and syrphid flies of *Episyrphus balteatus* (b) to fresh and old flowers of pollinated and unpollinated plants. Experiments were carried out 24 h after flowers had been pollinated. Pairwise comparison of treatments with generalized linear model with Poisson distribution. At least 30 insects of each sex were tested with 5-8 pairs of plants.

Effects of herbivory and pollination on volatile profile of *B. nigra* plants

Effects of herbivore infestation

The volatile profile of *B. nigra* was composed of benzenoids, terpenoids, fatty acid derivatives, and allyl isothiocyanate, the breakdown product of sinigrin, the major glucosinolate of *B. nigra*. Benzenoids and monoterpenoids made up 30% and 53% of the volatile blend, respectively. Upon herbivore infestation by *P. brassicae* caterpillars, *B. nigra* plants responded with changes in volatile emission. A PLS-DA (SIMCA P+ 12.0, Umetrics AB, Umeå, Sweden) including volatiles emitted by leaf-infested and control plants resulted in a model with 2 principal components; the first principal component explained 30% and the second principal component 40% of the total variance. From the PLS-DA it is clear that the volatile profile of leaf-infested plants is completely separated from the profile of control plants (Fig. 4). Flower-infestation induced changes in flower volatile emission. The PLS-DA shows that the volatile profile of flower-infested plants can be largely separated from the profile of uninfested control plants (Appendix C, Fig. C2) with 17% and 19% of the total variance being explained by the model obtained from the discriminant analysis.

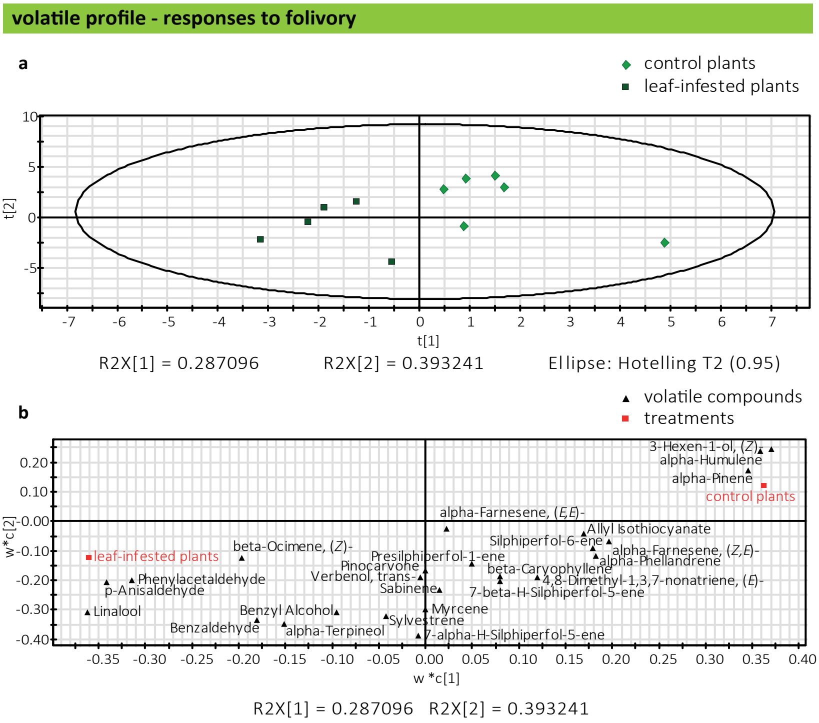

Fig. 4 Projection to Latent Structures Discriminant Analysis (PLS-DA) on volatile compounds collected from leaf-infested *Brassica nigra* plants, and from non-infested control plants. Plant volatiles were collected from aerial parts of plants 24 h after larvae of *Pieris brassicae* had hatched from the eggs. PLS-DA on the peak area of volatile compounds from headspace of *B. nigra* plants. a) Grouping pattern of samples according to the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; b) Contribution of individual volatile compounds to the first two principal components is shown in the loading plot of the PLS-DA components.

Effects of pollination and herbivory plus pollination

Pollination influenced the volatile profile of *B. nigra* plants, and so did the combined effects of pollination *plus* herbivory, when compared with the profile of control plants. The PLS-DA shows that the composition of the volatile profile of pollinated plants differed from that of unpollinated plants (Fig. 5). However, samples of plants subjected to leaf infestation plus pollination could neither be separated from samples of pollinated plants, nor from samples of unpollinated plants based on their volatile profile (Fig. 5). The first 2 principal components of the PLS-DA explained 47 and 16% of the total variance, respectively. Changes in the emission of terpenoids, for instance, contributed to these differences; the emission of terpenoids is induced in pollinated plants (Wilcoxon signed-rank test, P = 0.008) and in plants subjected to pollination plus leaf-infestation (Wilcoxon signed-rank test, P = 0.033).

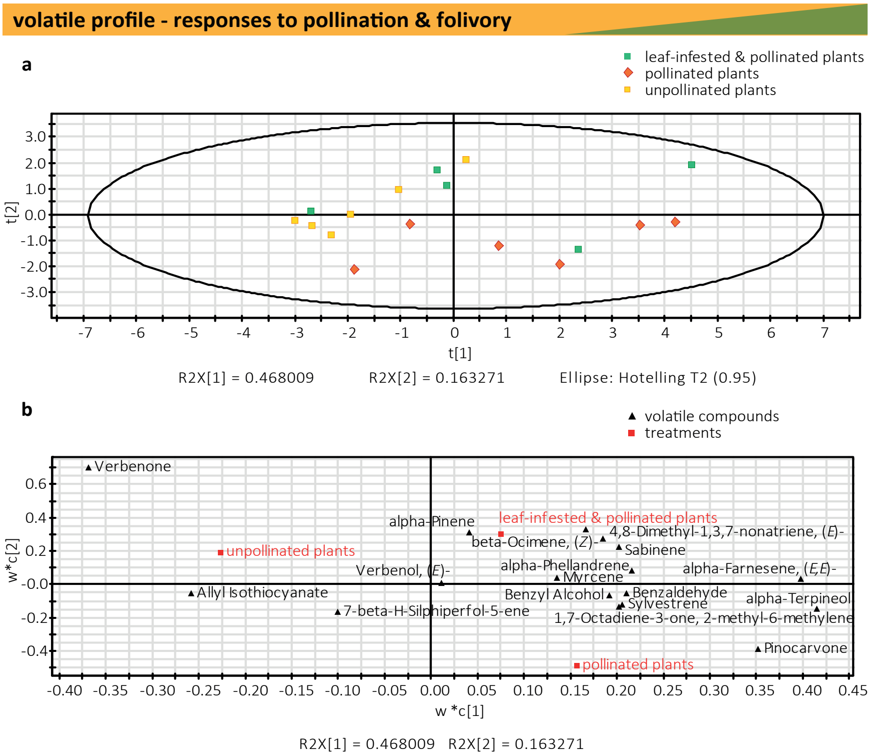

Fig. 5 Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile compounds collected from pollinated and unpollinated *Brassica nigra* plants and of plants that were both pollinated and infested with *Pieris brassicae* caterpillars. Plant volatiles were collected from aerial parts of plants 24 h after larvae of *Pieris brassicae* had hatched from eggs and 24 h after pollination. PLS-DA on the peak area (log-transformed data) of volatile compounds from headspace of *B. nigra* plants. a) Grouping pattern of samples according to the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; b) Contribution of individual volatile compounds to the first two principal components is shown in the loading plot of the PLS-DA components.

EFFECTS OF HERBIVORY AND POLLINATION ON THE NON-VOLATILE UHPLC PROFILE OF *B. NIGRA* LEAVES AND FLOWERS

Non-volatile profile of leaves and flowers

The UHPLC profile at 330 nm of the extracts of leaves of *B. nigra* plants is qualitatively and quantitatively different from that of flowers (Appendix C, Fig. C3). In this study, 55 compounds in leaf extracts and 92 compounds in flower extracts were quantified and compared on a relative basis. The maximum absorbance measured at 327 nm for the leaf extracts was 0.37, on average, and for the flower extracts this was 0.80 measured at 330 nm (Fig. C3).

Effects of herbivory on the phenolic content of *B. nigra* leaves and flowers

Herbivory quantitatively and qualitatively influenced the 330 nm UHPLC profiles of flowers. The wavelength of 330 nm was chosen as many of the major compounds appeared to be caffeic acid derivatives. Several other compounds were flavonols. Therefore, in the remainder of this paper we collectively indicate the non-volatile compounds present in the aqueous methanolic fraction as "phenolics". A PLS-DA was used to analyse the phenolic profiles of *B. nigra* leaves and flowers from leaf-infested, flower-infested and control plants at the same growth stages (Appendix C, Fig. C4). The phenolic profile of leaves was different from that of flowers, regardless of herbivore treatment or the time point investigated, as observed *via* discriminant analysis (Fig. C4), and from their UHPLC profiles (Fig. C3). The second principal component separated samples of flowers of infested plants from samples of flowers of non-infested control plants, at both time points tested (Figs. C3 & C4). From the PLS-DA, we observe that the first principal component largely separated the flower profile of leaf-infested plants from that of control plants (Fig. C4), whereas the phenolic profile of leaves of infested plants could not be distinguished from that of control plants. Similarly, when regarding flower-infested plants, changes after herbivory were restricted to flower tissues, and not apparent in leaf tissues (Fig. C4).

Effects of pollination and pollination plus herbivory on phenolic content of *B. nigra* leaves and flowers

The phenolic profile of pollinated flowers differed from that of unpollinated flowers and from that of plants that were subjected to pollination plus herbivory (Figs. 6 & 7). The first principal component of PLS-DA separated pollinated flowers from unpollinated flowers, whereas the second principal component separated the profile of flowers of infested from that of flowers of non-infested plants (Fig. 7). The phenolic profile of leaves of pollinated plants could also be distinguished from that of unpollinated plants and from that of plants that were subjected to pollination plus herbivory, based results of the discriminant analysis (Appendix C, Fig. C5).

Comparison of hand-pollination and insect-pollination methods

No difference was observed between hand-pollination and insect-pollination treatments. PLS-DA results show that samples of different plant parts subjected to hand-pollination clustered together with samples that were pollinated by the males of *P. brassicae* butterflies (Appendix C, Fig. C6).

Characterization of phenolic compounds

Glycosides of isorhamnetin, kaempferol and quercetin were detected among the main phenolic compounds of flower tissues of *B. nigra* (Appendix C, Table C1). Isorhamnetin dihexoside and kaempferol trihexoside were also detected in leaf tissues, apart from flower tissues (Table C1). The major constituents detected in flower tissues are most probably caffeic acid derivatives (Fig. 6); these are likely to be new natural products that are being further characterized by ^{1}H-NMR.

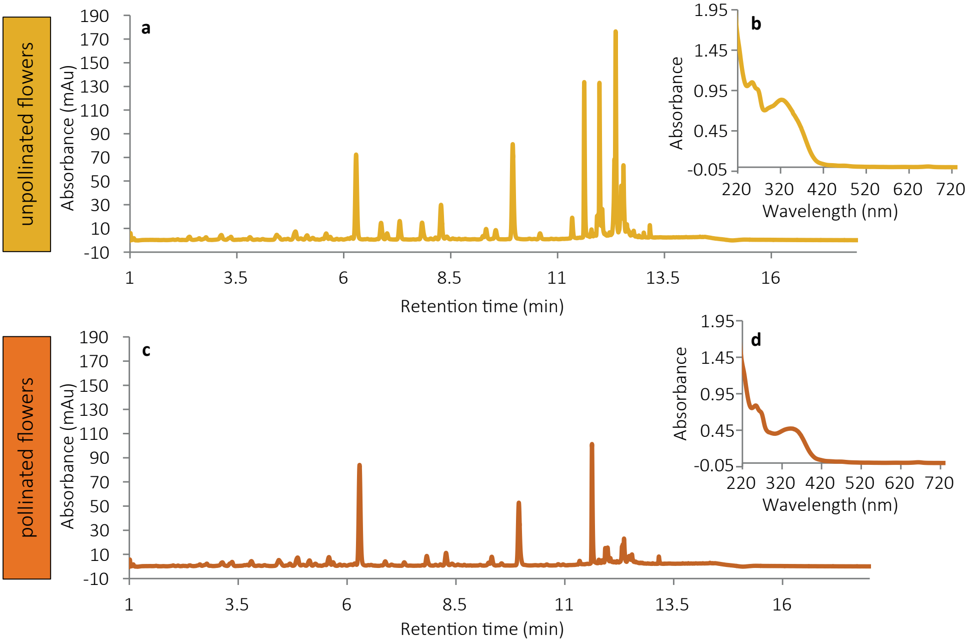

Fig. 6 Typical UHPLC profile at 330 nm of extracts of unpollinated flowers (a) and pollinated flowers (c) of *Brassica nigra* plants. Off-line UV-Vis spectra of the crude aqueous methanolic extracts of unpollinated (b) and pollinated flowers (d) are shown from 220 nm to 720 nm. The UV-Vis spectra show the average profile for 6 samples, in each case. Samples were harvested 24 h after flowers had been pollinated.

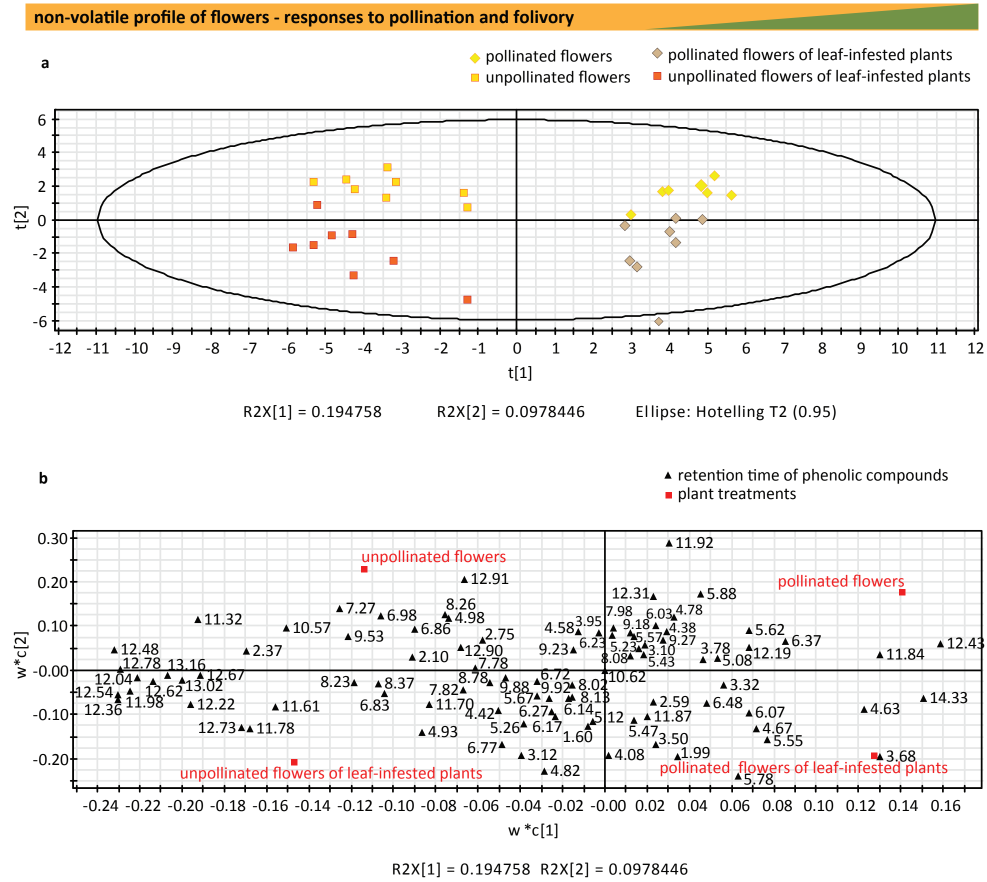

Fig. 7 Projection to Latent Structures Discriminant Analysis (PLS-DA) on non-volatile compounds of pollinated flowers and unpollinated flowers of pollinated *Brassica nigra* plants and of plants that were pollinated and infested with *Pieris brassicae* caterpillars. This UHPLC 330 nm profile is characteristic for plants parts 24 h after larvae of *Pieris brassicae* had hatched from eggs and 24 h after pollination. Projection to Latent Structure Discriminant Analysis (PLS-DA) on the peak area of individual compounds from flowers of *B. nigra* plants. (a) Grouping pattern of samples based on the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; (b) Loading plot of the PLS-DA components shows the contribution of individual compounds to the first two principal components. Numbers refer to the retention time in the UHPLC profiles.

UV-Vis absorbance spectra of leaves and flowers

In order to infer if changes in the UHPLC 330 nm profile of plant tissues in response to herbivory and pollination could be translated into colour changes of the plant extracts, the UV-Vis absorption spectra of the extracts of leaves and flowers subjected to different treatments were recorded. The UV-Vis profile of flower extracts of plants subjected to pollination is different from that of control plants and of plants that were exposed to herbivore infestation only (Appendix C, Fig. C7). A shift was observed in terms of wavelength and absorbance. For instance, in ultraviolet A (UVA; range 315–400 nm), pollinated flowers had a lower and shifted absorbance when compared with flowers exposed to the other treatments (Fig. C7).

Discussion

Our results show that *B. nigra* plants respond to herbivory and pollination with changes in the volatile and non-volatile chemical profiles of flowers. Butterflies respond to changes induced by pollination and herbivory, and syrphid flies to changes induced by florivory. The data suggest that herbivore-induced changes in plants may affect attraction of pollinators because such changes can interfere with local responses to pollination, influencing the cues exploited by flower visiting insects.

Plant responses to pollination are essential in optimizing reproductive success. Once a flower of a plant has been pollinated, even when the flower remains open for a few days longer, nectar production will be interrupted along with the production of scents and changes in visual cues used by pollinators (Weiss 1991, Luyt & Johnson 2002, Rodriguez-Saona *et al.* 2011). Pollinators indeed associate odours and visual cues with the reward offered by flowers (Burger *et al.* 2010, Rodriguez-Saona *et al.* 2011, Milet-Pinheiro *et al.* 2012). Plant responses induced by pollination are therefore local, *i.e.* only the flowers that have been pollinated will go through such changes and in this way pollinators are guided to unpollinated flowers of the same plant.

In our study, butterflies rarely visited pollinated flowers of *B. nigra* plants. This suggests that butterflies can perceive changes induced by pollination and avoid flowers that provide little rewards, as nectar production typically ceases after pollination. Syrphid flies, however, visited pollinated and unpollinated flowers at equal frequencies. *Episyrphus balteatus* mainly collects pollen from *B. nigra* flowers, and changes in nectar availability did not influence their behaviour. Syrphid flies could, however, distinguish between recently opened flowers and older flowers, which probably relates to the quality and/or quantity of pollen. Interestingly, we also observed that folivory influenced oviposition preference of *P. brassicae* butterflies, but not their flower feeding preference. The two pollinating insect species we studied exploited different cues depending on the resource they search for. In nature, *B. nigra* flowers are visited by many species of bees, syphids and butterflies. In such a generalized system, we expect that this diverse group of pollinators selects for diversity in flower chemistry and plasticity of responses to pollination. Herbivores, however, also influence flower and consequently influence pollinator behaviour (Kessler & Halitschke 2007, Lucas-Barbosa *et al.* 2011, Schiestl & Johnson 2013, Dormont *et al.* 2014).

Recent studies have observed that defensive responses to herbivores can positively and negatively affect pollinator behaviour and impact fruit and seed production by plants (Strauss & Murch 2004, Kessler *et al.* 2011, Swope & Parker 2012). In the context of the reproduction-defence trade-off, the production of herbivore-induced plant volatiles that attract carnivorous insects can conflict with attraction of pollinators that are essential for the plant reproductive success (Herms & Mattson 1992, Kessler & Halitschke 2009, Dicke & Baldwin 2010, Lucas-Barbosa *et al.* 2011). Results of this study show that not only induced responses to herbivory *per se* can influence pollinator behaviour, but can also interfere with plant responses to pollination. The volatile profiles of pollinated and unpollinated *B. nigra* plants differ distinctly according the discriminant analysis. However, when plants were exposed to both pollination and leaf-infestation, their volatile blends could not be distinguished, neither from the blends of pollinated nor unpollinated plants. Therefore, herbivory might affect pollinator preference because it can interfere with pollination-induced changes that are important in the mutualistic interaction between plants and pollinators.

Our results show that the UHPLC profile of leaves and flowers significantly changed in response to herbivory and pollination. Phenolic compounds play a role in plant defence against herbivores and are used as oviposition cues (Harborne & Grayer 1993). These compounds also colour flowers, and therefore, can play a role in pollinator attraction (Harborne & Grayer 1993). Both folivory and florivory induced changes in flower phenolic composition, but not in foliar phenolic composition of *B. nigra* plants. This resembles what we observed in terms of volatile emission by *B. nigra* leaves and flowers in our previous study; folivory by *P. brassicae* induced volatile production in flowers, but not in the leaves (Bruinsma *et al.* 2014). Thus, flowering *B. nigra* plants respond to herbivore attack with phytochemical changes in flowers, both in terms of flower volatiles and non-volatile phenolic compounds.

When regarding the effects of pollination, *B. nigra* plants responded by drastically down-regulating the levels of some of the phenolic compounds, more strongly so in response to pollination than in response to herbivory. Depending on the spatial distribution of the phenolic compounds within a flower petal (Svatos 2010, Miosic *et al.* 2013), changes induced by pollination can result in changes in the visual cues used by pollinators such as nectar guides or the overall flower colour. Moreover, changes in the biosynthesis of phenolic compounds may influence the biosynthesis of aromatic volatile compounds and *vice versa*, and therefore modify the colour-odour combinations that are used by pollinators (Dormont *et al.* 2014). Pollination also induced changes in the phenolic profile of leaves and whether this influences the behaviour of *P. brassicae* caterpillars remains to be investigated.

In conclusion, our study shows that plants respond to herbivory and pollination with changes in the flower odours and pigments, which can influence the specificity of colour-odour combinations that shape the behaviour of flower visitors. Systemic responses to herbivory can interfere with local responses to pollination that are essential in maximizing plant fitness. We conclude that both herbivores and pollinators induce important phenotypic changes in flowers and thus, these responses must be examined in concert because in nature plants are exposed to herbivores and pollinators at the same time.

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Chapter 6

Caught between parasitoids and predators – survival of a specialist herbivore on leaves and flowers of mustard plants

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Abstract

The survival of insect herbivores is typically constrained by food choice and predation risk. Here, we explored whether movement from leaves to flowers increases survival of herbivores that prefer to feed on floral tissues. Combining field and greenhouse experiments, we investigated whether flowering influences the behaviour of *Pieris brassicae* butterflies and caterpillars and, consequently, herbivore survival in the field. In this context, we investigated also if flowers of *Brassica nigra* can provide caterpillars refuge from the specialist parasitoid *Cotesia glomerata* and from predatory social wasps. By moving to flowers, caterpillars escaped from the parasitoid. Flowers are nutritionally superior when compared with leaves and caterpillars develop faster when feeding on flowers. However, late-stage caterpillars can be intensively preyed upon by social wasps, irrespective of whether they feed on leaves or flowers. We conclude that flower preference by *P. brassicae* is more likely driven by nutritional advantages and reduced parasitism on flowers, than by risks of being killed by generalist predators.

Keywords: florivory, foraging behaviour, herbivore-induced plant volatiles, parasitic wasps, social wasps.

Introduction

The survival of invertebrate animals that feed on sessile organisms is typically constrained by food choice and predation risk (Jaenike 1990, Ohsaki & Sato 1994, Berdegue & Trumble 1996, Murphy 2004, Choh & Takabayashi 2007, Kumagai 2008). Examples of ecological specialization driven by food choice and predator avoidance can be found in terrestrial and marine environments, and the contribution of either factor to ecological specialization is often difficult to disentangle (Singer *et al.* 2004a, Kumagai 2008). Plants, for instance, can serve as food source as well as refuge from predators for invertebrate herbivores (Schoonhoven *et al.* 2005, Townsend 2012). Individual survival may be influenced by choices based on food quality and risks of predation and parasitism (Lima & Dill 1990, Dicke & Grostal 2001, Schoonhoven *et al.* 2005).

Ideally, to survive insect herbivores need to select high quality food and, at the same time, avoid predators and parasitoids (Lima & Dill 1990, Dicke & Grostal 2001, Schoonhoven *et al.* 2005). Food quality is determined by nutrient content, physical properties such as leaf toughness, and plant secondary metabolites (Schoonhoven *et al.* 2005). Within a plant, herbivorous larvae can selectively feed on plant tissues on which they can develop faster. Plant tissues that contain high concentrations of constitutive and inducible secondary metabolites, such as young leaves, are frequently preferred by specialist herbivores and avoided by generalists (Kursar *et al.* 2006, Perkins *et al.* 2013). Herbivores can also selectively feed on plants on which predation risk is lower. The survival of caterpillars of the Alaskan swallowtail butterfly *Papilio machaon aliaska,* for instance, is greater on *Artemisia arctica* and on *Petasites frigidus*, although the growth rate of caterpillars is higher on the ancestral host plant *Cnidium cnidiifolium* (Murphy 2004). Thus, to survive swallowtail butterflies favour host plants on which risk of predation is lower at the cost of reduced growth rate. Insect herbivores may indeed choose suboptimal host plants or parts of plants if they can escape there from their natural enemies (Ohsaki & Sato 1994, Gross *et al.* 2004, Mulatu *et al.* 2004, Murphy 2004, Singer *et al.* 2004a).

Flowers can be good food for herbivores. Floral traits that primarily make plants attractive to pollinating insects (Raguso 2008), may render plants also more attractive to adult herbivores searching for host plants (Bronstein *et al.* 2009, Reisenman *et al.* 2010, Agerbirk *et al.* 2011). Herbivores might prefer to feed on flowers rather than on leaves of plants (McCall & Irwin 2006), even when they do not nutritionally benefit from feeding from flowers (Matter *et al.* 1999, Agerbirk *et al.* 2011). For instance, food quality did not explain population-level responses of the milkweed beetle *Tetraopes tetraophthalmus* to milkweed flowers (Matter *et al.* 1999). Floral traits may influence the perception of plants by carnivorous insects that search for prey and hosts, particularly when flowers have been damaged by herbivores (Jonsson & Anderson 2008, Dannon *et al.* 2010). Carnivorous insects can use odours produced by plants infested with herbivores to locate their prey or host. The large majority of these studies have focused on plants in the vegetative stage (Mumm & Dicke 2010, Lucas-Barbosa *et al.* 2011). Herbivore damage to leaves cannot only lead to changes in volatile emission by leaves, but may also result in changes in volatile emission by flowers (Röse & Tumlinson 2004, Effmert *et al.* 2008, Zangerl & Berenbaum 2009), and damage to flowers can trigger the production of herbivore-induced plant volatiles in leaves (Röse & Tumlinson 2004). Changes in flower odours can influence the behaviour of natural enemies of the herbivores. For instance, parasitoids of the cowpea pod borer *Maruca vitrata* and the pollen beetle *Meligethes aeneus* use odours emitted by herbivore-infested flowers to locate their herbivorous host; these herbivores develop exclusively on reproductive tissues of their host plants (Jonsson & Anderson 2008, Dannon *et al.* 2010). Thus, flower odours can mediate predator-prey interactions.

Among herbivores that specialize on brassicaceous plants, individuals of several species move towards flowers to feed. For instance, caterpillars of the lepidopterans *Pieris brassicae*, *P. rapae*, *Plutella xylostella*, *Anthocharis cardamines* and the sawfly *Athalia rosae* become florivorous during their development (Smallegange *et al.* 2007, Bandeili & Müller 2010, Agerbirk *et al.* 2011). *Pieris brassicae* caterpillars and *A. rosae* sawfly larvae develop faster when feeding from flowers than from leaves of plants in the flowering stage (Smallegange *et al.* 2007, Bandeili & Müller 2010). In contrast, food quality does not explain why *A. cardamines* caterpillars prefer to feed from flowers (Agerbirk *et al.* 2011).

When herbivores prefer to feed on flowers and develop faster on these tissues, we would expect that movement to flowers would increase survival rate of these herbivores (Benrey & Denno 1997). The survival chance of herbivores can be driven not only by the quality of the food they consume, but also by the likelihood of being killed by their natural enemies (Ohsaki & Sato 1994, Singer *et al.* 2004a, Kumagai 2008). The assumption that choices based on food quality and predator avoidance can increase survival underlies the slow growth - high mortality hypothesis. This hypothesis predicts that when insect larvae develop faster, the time window in which they are exposed to their natural enemies is reduced and, consequently, herbivore fitness is increased (Benrey & Denno 1997). However, herbivores are exposed to multiple enemies, and a prolonged development does not always correlate with higher vulnerability to natural enemies (Clancy & Price 1987, Williams 1999, Lill & Marquis 2001).

In this study, we combined field and greenhouse experiments to investigate whether flowers can provide caterpillars with refuge from their natural enemies and what are the consequences for the survival of *P. brassicae* when feeding on flowering plants of the annual plant *Brassica nigra*. More specifically, we have investigated: a) whether flowering influences the behaviour of adult herbivores and caterpillars; b) whether caterpillars survive better when feeding on leaves or on flowers of *B. nigra* plants; c) whether herbivore larvae are more frequently attacked by their specialist parasitoid when feeding from leaves or flowers; and lastly d) how predation rates of caterpillars by generalist predators are influenced by the feeding site selected by the larvae.

Materials and methods

STUDY SYSTEM

Pieris brassicae L. (Lepidoptera: Pieridae) is a specialist herbivore of plants in the family Brassicaceae. This lepidopteran is a gregarious species and female butterflies lay batches of up to 100 eggs on the underside of leaves. When eggs hatch, caterpillars initially feed gregariously on leaves of a flowering *B. nigra* plant, but second-instar larvae soon move to flowers. In The Netherlands, *P. brassicae* caterpillars are frequently attacked by the gregarious parasitoid *C. glomerata* L. (Hymenoptera: Braconidae) and by social wasps, such as *Polistes dominula* (Christ), *Vespula germanica* (Fabr.) and *V. vulgaris* (L.). *Cotesia glomerata* is an endoparasitoid and can successfully complete development into adult wasps when parasitizing first or second instar larvae of *P. brassicae* (Geervliet & Brodeur 1992). Social wasps forage for water, plant fibres, carbohydrates and animal protein. These wasps are generalist predators and forage for animal protein to feed their progeny (Richter 2000).

Brassica nigra is an annual plant that belongs to the brassicaceous family. In the Netherlands, this wild species grows as early successional plant and stands in high densities.

INSECTS AND PLANTS

Pieris brassicae used in the experiments were obtained from a laboratory colony reared on Brussels sprouts (*Brassica oleracea* var. *gemmifera*) plants in a climate room (22 ± 1 °C, 50-70% r.h., L16:D8). The adults were provided with a 10% sucrose solution as food. The parasitic wasp *C. glomerata* was reared on *P. brassicae* caterpillars of the laboratory colony. The parasitoid colony was maintained in a greenhouse compartment (25 ± 1 °C, 50-70% r.h., L16:D8), and adults were kept in climate cabinets (25 ± 1 °C, L16:D8). Honey and water were provided to the adult wasps. The parasitoids were used for the experiments after 4 to 7 days since eclosion from cocoons.

Seeds of an early-flowering accession (CGN06619) of *B. nigra* were obtained from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands) and multiplied by exposing plants to open pollination in the surroundings of Wageningen. Seeds collected from 25 plants were mixed to obtain seed batches for the experimental plants. For the greenhouse experiments, potted *B. nigra* plants were reared in a greenhouse compartment (23 ± 2 °C, 50-70% r.h., L16:D8). For the field experiments, *B. nigra* potted plants were reared outside on tables protected by insect screen, in a location close to the field site. Plants in the flowering stage, with several flowers open, and in the vegetative growth stage, *i.e* stages 4.2 and 3.1, respectively based on classification for *Brassica napus* (Harper & Berkenkamp 1975) were used for the experiments.

PLANT TREATMENTS

For the herbivore-infestation treatment, flowering plants with a few open flowers were infested with one egg clutch of *P. brassicae* by exposing plants to butterflies in an oviposition cage (100 cm x 70 cm x 82 cm). While the plant was inside the oviposition cage, flowers were covered with a mesh bag to prevent being visited by the butterflies. The number of eggs on a plant was reduced to thirty by gently removing surplus eggs shortly after the plants had been removed from the oviposition cage.

FORAGING BEHAVIOUR, DISPERSAL AND SURVIVAL OF THE HERBIVORE *PIERIS BRASSICAE* ON FLOWERING *BRASSICA NIGRA* PLANTS

Effects of flowering on host plant selection by butterflies

greenhouse experiment To test whether flowers influence host-plant selection by *P. brassicae* female butterflies, a choice between *B. nigra* plants in the flowering and vegetative stages was offered to mated female butterflies. Experiments were carried out in a flight chamber set up (gauze tent of 293 cm × 200 cm × 230 cm in height) in a greenhouse compartment (25 ± 2 °C, 50-70% r.h.). The preference of sucrose-fed adult butterflies was tested. First landing and oviposition preference were scored during the bioassays. A single butterfly was released at a time, at a distance of 85 cm from the plants. When a butterfly did not make a choice within 15 minutes, it was recorded under 'no response'. Fifty adult butterflies were individually tested over 5 days. On each day, different groups of plants were offered to the butterflies. Butterflies were used in the experiments at 5 to 7 days after eclosion and two days after mating. They were considered to be naïve because they had not been exposed to a plant as an adult prior to the bioassays. Each individual butterfly was used only once and then discarded. The positions of the two groups of plants were interchanged after every 3 butterflies tested to compensate for possible positional bias.

Effects of flowering on foraging behaviour of caterpillars – greenhouse experiment

In a greenhouse experiment (23 ± 2 °C, 50-70% r.h., L16:D8), we tested whether movement of caterpillars to the flowers is driven by geotaxis only or whether caterpillars orient to flowers. Ten plants with a few open flowers (growth stage 4.2) were infested with *P. brassicae* eggs as previously described. On the day of larval hatching, five of these plants were suspended upside down by two cords. The other five plants were kept in the normal upright position, and regarded as control plants. The soil of control and test plants was covered with a mesh bag. After the larvae had hatched from the eggs, the position of the caterpillars on leaves, stem or flowers was recorded after one, three, five and seven days.

Dispersal and survival of caterpillars in the field

In a common garden experiment, we assessed caterpillar survival and dispersal within plots of *B. nigra* plants. Field layout for trials 1 and 2 consisted of 16 plots of flowering *B. nigra* plants infested with *P. brassicae* and 16 control plots, and was laid out as described by (Lucas-Barbosa *et al.* 2013). Caterpillar dispersal and survival were assessed by counting the number of individuals present on each of the five plants per plot and determining their position on (1) a leaf, (2) a flower/bud, or (3) stem, every other day. At the end of the experiments, when plants were harvested, caterpillars found on the plants were collected and frequency of parasitism estimated according to (Lucas-Barbosa *et al.* 2013). We carried out two serially repeated trials that lasted for about a month each, in June and July of 2012, at an experimental field site in Wageningen. Kaplan-Meier curves were made for survival data of early and late instars caterpillars. Plots of infested plants were randomly assigned to the early or late instar group, to ensure independence of these groups. The total time span of the early instar group was defined to range from 0 - 192 h after larvae had hatched, *i.e* the period in which most herbivores were feeding on leaves. The late instar group was defined to range from 216 h since larvae had hatched till the end of the experiment, *i.e* the period in which most herbivores were feeding on flowers. A log-rank test was used to compare the survival curves.

PARASITISM RATES OF CATERPILLARS BY A SPECIALIST PARASITOID ON FLOWERING *BRASSICA NIGRA* PLANTS

Parasitism rates in a natural plant population in the field

To test whether flowering and non-flowering plants influence the behaviour of parasitoids while searching for hosts in nature, we estimated parasitism rates of *P. brassicae* by *C. glomerata* throughout the growing season of the plants, during 15 consecutive weeks in the field. In the first week of observations all plants were in the vegetative stage. In the last week, all plants were flowering. Every week we randomly selected 20 *B. nigra* plants from a natural population (nature reserve Blauwe Kamer, Wageningen) and infested these plants with 10 second instar *P. brassicae* caterpillars. The caterpillars were recollected 48 h later and dissected in the laboratory to assess whether they were parasitized by *C. glomerata*. Using a stereomicroscope, we recorded the number of parasitized caterpillars and counted parasitoid eggs inside each caterpillar.

Foraging behaviour of parasitic wasps towards and on leaf- and flower-infested plants – greenhouse experiment

To test whether folivory and florivory by *P. brassicae* caterpillars affect the foraging behaviour of the parasitic wasp *C. glomerata*, preference of adult female wasps was studied. We tested preference of wasps for leaf-infested plants and flower-infested plants *versus* non-infested plants. Preference of *C. glomerata* was tested 72 h after caterpillars had hatched from the eggs. At this time point, caterpillars can be found feeding from leaves or from flowers of *B. nigra* plants (23 ± 2 °C, 50-70% r.h.), and are suitable for parasitism by *C. glomerata*. For the flower-infestation treatment, caterpillars were free to move to flowers. For the leaf-infestation treatment we prevented caterpillars from moving to the flowers by placing cotton wool around the petiole. Cotton wool was removed from all plants before the behavioural experiments. These experiments were carried out using the same set-up and conditions described for the experiments with butterflies (above). We released non-experienced female parasitoids individually and recorded first choice to an infested or non-infested plant, and whether the wasp landed first on a leaf or on a flower. When a wasp did not make a choice within 10 min it was recorded under 'no response'. Each individual wasp was used only once and then discarded. Additionally, we tested the preference of wasps for leaf-infested plants *versus* flower-infested plants. In this case, we recorded not only wasps' first choice to plants of the two treatments, but also the time wasps spent on the leaves or on the flowers of plants of the two treatments, and the number of oviposition events. Each observation for a single wasp lasted for 10 min, and was recorded using a handheld computer (Psion Workabout Pro^{TM} 3, London, UK), programmed with The Observer software (version 10, Noldus Information Technology, Wageningen). The position of plants was interchanged after every 3 wasps tested to compensate for possible positional bias. For each of the three pairs of treatments, we tested up to 12 wasps per pair of plants, and at least 6 pairs of plants per treatment.

PREDATION OF CATERPILLARS BY PREDATORY SOCIAL WASPS ON FLOWERING *BRASSICA NIGRA* PLANTS

Foraging behaviour of predatory social wasps in the field

In the same field set-up used to investigate survival of the herbivores, we observed the foraging behaviour of naturally occurring social wasps in *B. nigra* plots infested with *P. brassicae* caterpillars and on plants in control *B. nigra* plots. Throughout the development of the herbivores, we recorded the presence or absence of social wasps in our common garden set-up. At fixed time intervals we walked in between the plots for 5 min, and recorded the presence of social wasps in the field, and observed predation of caterpillars or flower visitors by these wasps. All plots were observed 5 times per day (between 9 am and 5 pm), when weather conditions were suitable (17 - 25 °C; wind speed $\leq$ 6 m s^{-1}). During this period, we recorded the duration of the time that social wasps spent in *B. nigra* plots infested with *P. brassicae* caterpillars and in control *B. nigra* plots. Wasp behaviour was recorded with a handheld computer (Psion Workabout, Pro^{TM} 3, London, UK) programmed with The Observer XT software (version 10, Noldus Information Technology, Wageningen, The Netherlands). We observed one wasp at a time, and each observation was finished when the wasp had either left the field or was lost, *i.e* out of sight of the two observers. The time that wasps spent in a plot was defined as walking on plants of a plot or on the ground below the plants, and flying around the plot. Additionally, when the wasp initiated an attack, the type of prey (caterpillar, pollinator or other) and time that it spent handling prey were recorded. Species identity of the wasps was recorded. These experiments were carried out in two different trials during the season, in July and in August of 2012.

Predation rates of caterpillars by social wasps on leaves and flowers in the field

In a manipulative field experiment, we investigated whether *P. brassicae* caterpillars were more preyed upon when they were feeding on leaves or flowers of *B. nigra* plants. Field layout was similar to the one used to investigate herbivore survival. In total, sixteen plots of five flowering plants each were established in the field; half of the plots were infested with *P. brassicae* eggs (infestation procedure described above) and half were non-infested control plots. Caterpillars that had hatched from the eggs developed on the plants until they reached the fourth larval instar. In this way we induced plants to respond to eggs and herbivory simulating what occurs in nature. Caterpillars that survived till their fourth larval instar were then collected and discarded. From this moment onwards, plants were infested daily in the morning with 10 late-instar caterpillars (L4 or L5) from the *P. brassicae* culture. In half of the plots, later instar caterpillars were placed on leaves and in the other half of the plots on the flowers of *B. nigra* plants. To estimate predation rates of caterpillars on flowers and leaves, numbers of caterpillars were counted in the late afternoon and the remaining caterpillars were discarded. We repeated this for seven consecutive days. The location on which caterpillars were placed was switched between leaves and flowers every day to prevent that predators learned where to locate these prey. We did two trials of these experiments: in June and in August 2012.

Results

OVIPOSITION PREFERENCE OF BUTTERFLIES FOR FLOWERING PLANTS OVER VEGETATIVE PLANTS

In a two-choice setup in the greenhouse *Pieris brassicae* butterflies preferred to land first on flowering *B. nigra* plants (GLM with binomial distribution, $P < 0.001$) and favoured these plants for egg deposition over plants in the vegetative stage (GLM with binomial distribution, $P = 0.020$) (See supporting information in Appendix D, Fig. D1). No inter-day variation regarding butterfly responses to pairs of plants were observed (GLM with binomial distribution, first landing: $P = 0.993$ and oviposition: $P = 0.811$). Eggs were laid preferentially on true leaves of flowering plants when compared to leaves of the inflorescence (binomial test, $P < 0.001$).

FORAGING BEHAVIOUR OF CATERPILLARS

Second instar larvae of *P. brassicae* move to flowers of *B. nigra* plants. When plants were in the upright position most caterpillars were found feeding from flowers 5 days after they had hatched from the eggs (Appendix D, Fig. D2). When plants were positioned upside down, it took on average 2 days longer for caterpillars to reach the flowers, because caterpillars first moved up (to the pot), and subsequently down to the flowers (Fig. D2, ANOVA repeated measures, $P = 0.003$). In this situation, most third instar larvae were found on the flowers on day 7 (Fig. D2).

DISPERSAL AND SURVIVAL OF CATERPILLARS IN THE FIELD

In the field, *P. brassicae* eggs hatched after approximately 14 days in trial 1 and after 9 days in trial 2. Mean temperatures for trials 1 and 2 were 15 °C and 18 °C respectively. Egg survival was higher (69%) during trial 1 than during trial 2 (53%) (Chi square test, df = 1, $P = 0.025$). Some hours after hatching from the eggs, *P. brassicae* caterpillars started feeding from leaves on which they had hatched (Appendix D, Fig. D3). After 120 h, they were found feeding on flowers and with time, the proportion of caterpillars feeding on flowers increased (Fig. D3). Caterpillars also moved to neighbouring plants in increasing numbers over time (Fig. D3). Late instar *P. brassicae* caterpillars had a lower chance of survival than early instars, an effect that was observed for both trial 1 (Fig. 1a, Log rank test, $P < 0.001$) and trial 2 (Fig. 1b, Log rank test, $P < 0.001$). The main predators of early instar larvae were ladybird beetles (Coccinellidae). Late instar larvae were extensively preyed upon by social wasps. Of the caterpillars collected at the end of trial 1, 79% was parasitized. For trial 2, only three caterpillars could be retrieved from the field at the end of the trial and, therefore, parasitism rate was not estimated.

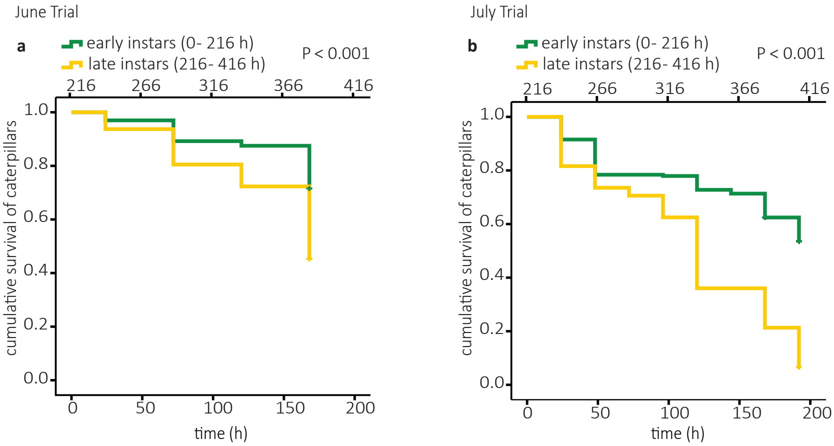

Fig. 1 Cumulative survival of early and late instars of *P. brassicae* caterpillars feeding on *B. nigra* plants over time, for June (a) and July (b) trials. Log rank test was used to compare survival of early instar larvae with that of later instar larvae, for June and July trials of 2012.

PARASITISM RATES OF CATERPILLARS BY A SPECIALIST PARASITOID ON FLOWERING *BRASSICA NIGRA* PLANTS

In the field, we observed parasitism of *P. brassicae* caterpillars over the entire growth season of a natural population of *B. nigra* plants. A peak of parasitism was found around the time that the plant population started flowering (Fig. 2).

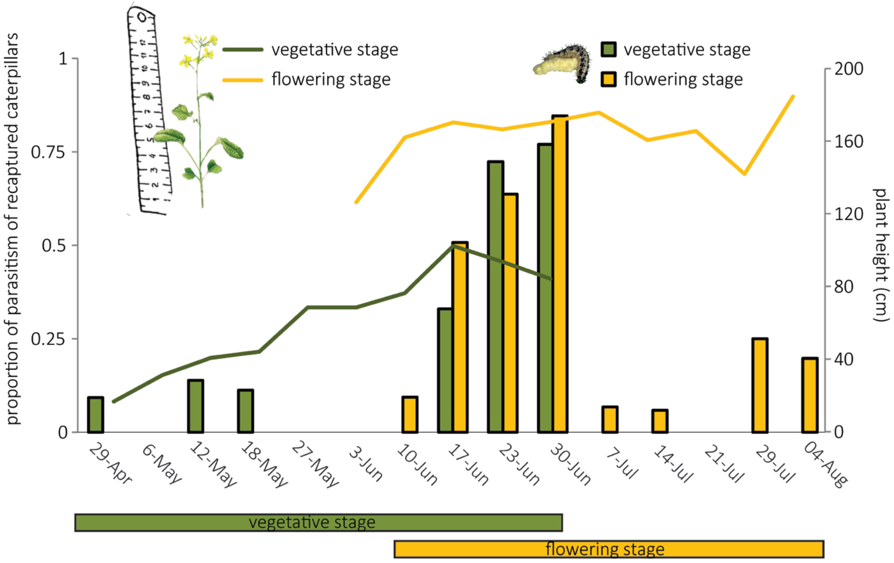

Fig. 2 Proportion of *Pieris brassicae* caterpillars parasitized by *Cotesia glomerata* wasps recaptured from *Brassica nigra* plants and height of plants in the vegetative and flowering stages. Proportion of recaptured caterpillars and plant height is shown for a natural population of *B. nigra* plants, in nature reserve Blauwe Kamer near Wageningen, The Netherlands.

During the behavioural experiments in the two-choice setup in the greenhouse, *C. glomerata* landed more often on flowering *B. nigra* plants infested with *P. brassicae* caterpillars than on non-infested flowering plants, irrespectively of whether caterpillars were feeding from leaves (Fig. 3, binomial test, $P = 0.011$) or from flowers (Fig. 3, binomial test, $P < 0.001$). Sixty-seven per cent of the wasps landed first on a leaf, irrespective of caterpillars being present on a leaf or on flowers of an infested plant (Fig. 3). *Cotesia glomerata* landed more frequently on leaf-infested plants than on flower-infested plants (Fig. 4a, binomial test, $P = 0.022$). Once they had landed on a plant, wasps spent more time on leaves than on flowers of that plant (Fig. 4b, Wilcoxon's matched-pairs signed rank test, $P = 0.001$ and Fig. 5). *Cotesia glomerata* parasitized hosts more frequently on leaf-infested plants than on flower-infested plants (Fig. 4c, Mann-Whitney U test, $P = 0.018$).

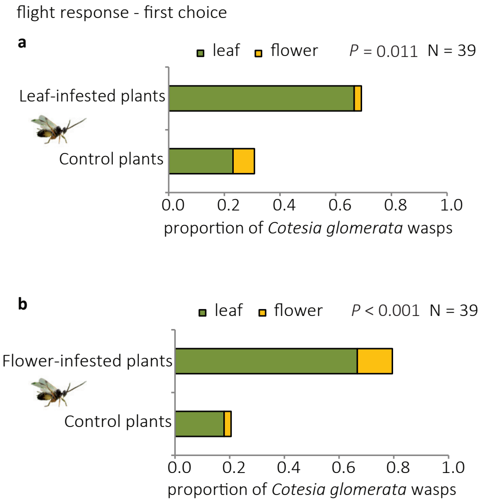

Fig. 3 Proportion of *Cotesia glomerata* wasps that landed first on either a flower or a leaf of plants infested with second instars of *Pieris brassicae* caterpillars or on non-infested plants. a) Caterpillars were feeding on the leaves of the infested plants; b) Caterpillars were feeding on the flowers of infested plants. Pairwise comparison of treatments with binomial test. N = number of insects that responded.

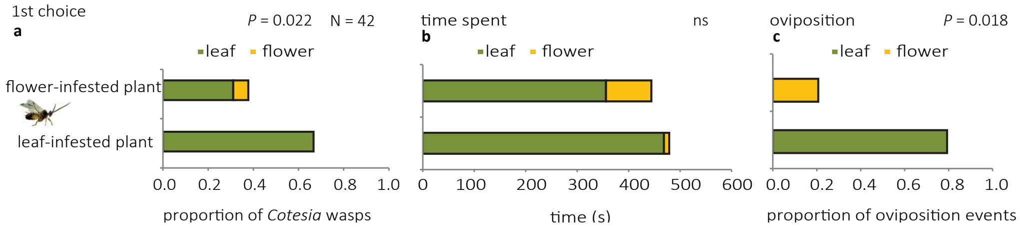

Fig. 4 Preference of *Cotesia glomerata* wasps for plants with second instars of *Pieris brassicae* caterpillars feeding on leaves versus plants with caterpillars feeding on flowers. a) Proportion of *C. glomerata* wasps that landed first on a flower or on a leaf of leaf-infested plants and of flower-infested plants. Pairwise comparison of treatments with binomial test; b) Mean time spent by the wasps on the leaves or flowers of plants of the two treatments; c) Proportion of oviposition events observed on the plants of the two treatments. Pairwise comparison of treatments with Mann-Whitney-U test. N = number of insects that responded to the test.

Fig. 5 *Pieris brassicae* caterpillars under attack of a parasitic wasp *Cotesia glomerata* (a); caterpillars of *P. brassicae* on a stem (b) and on flower buds (c) of *Brassica nigra* plants. Photograph credits: Dani Lucas-Barbosa.

PREDATION OF CATERPILLARS BY PREDATORY SOCIAL WASPS ON FLOWERING *BRASSICA NIGRA* PLANTS

Foraging behaviour of social wasps in the field

Social wasps were only observed foraging in the field plots when *P. brassicae* caterpillars were L4 or larger. Therefore, we only recorded the foraging behaviour of the wasps in infested and non-infested plots when plants carried late instar caterpillars (L4 or L5) for each of the two trials. Two species of social wasps were predominantly observed preying on caterpillars during the field experiment: *V. germanica* and *P. dominula* (Fig. 6). Because species of the genus *Vespula* are difficult to distinguish in flight, records of these wasps were noted as *Vespula* sp. When we were able to identify a *Vespula* sp. wasp, the species was always identified as *V. germanica*. During the July trial, both *Vespula* sp. and *P. dominula* spent more time foraging in *B. nigra* plots infested with caterpillars than in control plots of plants (Appendix D, Fig. D4, Wilcoxon matched pairs, $P < 0.001$ and $P = 0.003$ respectively). *Polistes dominula* spent more time handling caterpillars than did *Vespula sp.* (Fig. D4, Mann-Whitney U-test, $P = 0.009$). *Vespula* sp. wasps were often observed stealing parts of prey that were previously killed by *P. dominula* or by other *Vespula* species and they can also share prey with their conspecifics (Fig. 6e). Both wasp species removed the gut of the caterpillar, and cut prey in parts that were made into a ball shape before being carried back to the nest (Fig 6d and video link in ESM). During the July trial, both species were only observed attacking *P. brassicae* caterpillars. During the August trial, *Vespula* sp. was observed flying within the experimental plots of plants and mostly pursuing pollinators. No clear preference for infested plots was observed in this trial (Fig. D4, Wilcoxon matched pairs, $P = 0{,}057$). The attack success ratio was low; out of the 43 wasps observed only 2 successfully killed an insect pollinator (5%), in both cases an undetermined species of syrphid fly.

Fig. 6 Social wasps *Polistes dominula* and *Vespula germanica* with prey. *Polistes dominula* attacking and handling prey until *Pieris brassicae* caterpillar is made into a ball that can be carried by the wasp to the nest (a, b and d). *Vespula germanica* attacking a syrphid fly (c) and sharing *P. brassicae* larva with another wasp of the same species (e). Photograph credits: Yavanna Aartsma and Dani Lucas-Barbosa.

Predation rates of late-stage caterpillars by social wasps on leaves and flowers in the field

In a manipulative field experiment, we tested whether survival rates of late-stage *P. brassicae* caterpillars was higher on leaves or on flowers. Mortality of caterpillars placed on leaves and flowers was equal, both per day and on average (Fig. 7).

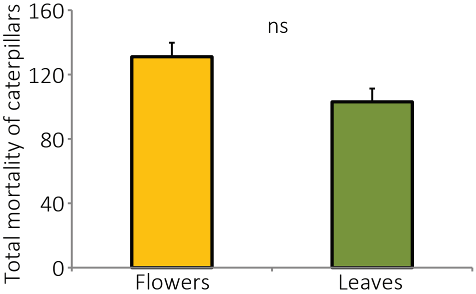

Fig. 7 Total mortality of fifth instar larvae of *Pieris brassicae* on leaves and flowers of *Brassica nigra* plants. Data analyzed with Student's t-test. Total number of caterpillars preyed upon is shown for 2 trials.

Discussion

By moving to flowers of *B. nigra* plants, *P. brassicae* caterpillars escaped from their parasitoid *C. glomerata*. However, although the inflorescence of *B. nigra* may provide a refuge to early instar larvae, late instar caterpillars can be intensively preyed upon by generalist predators, as we observed in our field experiments. Social wasps caused a high level of mortality of caterpillars when the late-stage larvae fed either on flowers or on leaves. Thus, movement to the flowers does not prevent caterpillars from being preyed upon by these generalist predators. Flowers are, however, nutritionally superior and caterpillars develop faster when feeding on flowers than when feeding on leaves (Smallegange *et al.* 2007). Therefore, we conclude that by moving to flowers caterpillars reduce chances of being attacked by *C. glomerata* and increase their chances of reaching the reproductive stage by feeding on flower tissues that support faster development. We next discuss how flowers affect parasitoid-host and predator-prey interactions and consequences for the survival of the herbivores.

Flowers of *B. nigra* plants remarkably influence herbivore behaviour and the interactions of the herbivore with its specialist parasitoid. Plants in the vegetative and flowering stages produce different odours (Lucas-Barbosa *et al.* 2011). Flowering plants may respond differently to herbivory when compared to plants in the vegetative stage (Hare 2010, Diezel *et al.* 2011). Indeed responses of flowering plants to herbivory can influence host-plant selection by adult herbivores (Reisenman *et al.* 2010), interactions with pollinators (Poveda *et al.* 2003, Kessler & Halitschke 2009, Kessler *et al.* 2011), and predator-prey interactions (Jonsson & Anderson 2008, Pareja *et al.* 2012). In nature, *C. glomerata* wasps respond to cues that indicate the presence of their hosts. These cues can be emitted by plants in different developmental stages. *Cotesia glomerata* use herbivore-induced plant volatiles emitted by plants in the vegetative stage to locate first and second instar *P. brassicae* caterpillars (Mattiacci *et* *al.* 1994). In a natural population of *B. nigra* plants, we observed that host-infested *B. nigra* seedlings may attract parasitoids. However, it is important to note that peak parasitism of *P. brassicae* caterpillars by *C. glomerata* wasps coincides with the onset of flowering. Therefore, parasitoids are exposed to odours emitted by plants in the flowering stage that carry hosts.

Host-plant selection in Lepidoptera is mainly determined by the adult herbivore; the winged adult is more mobile than the caterpillars. In the present study, host-plant selection by *P. brassicae* was influenced by flowers. Adult *P. brassicae* preferred to lay eggs on leaves of plants that were flowering over those of plants in the vegetative stage. Their caterpillars indeed prefer to feed on flowers over leaves (Smallegange *et al.* 2007). Flowers of *B. nigra* are better food than leaves for these herbivores, even if flowers contain 5 times more glucosinolates than leaves (Smallegange *et al.* 2007). By hanging plants upside down, we observed that, although negative geotaxis was strong, second instar caterpillars avoided leaves of a flowering plant, and searched for flowers on which their growth and development is faster (Smallegange *et al.* 2007). *Pieris brassicae* caterpillars do not only develop faster when feeding on flowers, but also experience enemy-reduced space against its most common foe, the parasitic wasp *C. glomerata*. Our results clearly show that parasitoid wasps were arrested by caterpillar-damaged leaves because wasps spent most time on the leaves even when all caterpillars were already feeding on flowers. It is interesting to note, however, that leaf-feeding by *P. brassicae* caterpillars mainly induces changes in the flower volatile blend, and not in volatiles emitted by leaves of *B. nigra* plants (Bruinsma *et al.* 2014). Therefore, we speculate that *C. glomerata* perceive changes in volatiles emitted by flowers to locate a caterpillar-infested plant, and subsequently land preferentially on leaves perhaps by exploiting visual cues, such as the green colour of leaves. We expect selection on parasitoids to arrive at plants as soon as herbivore larvae hatch from the eggs (Fatouros *et al.* 2012), and for caterpillars to move even sooner to flowers because in this way they can escape parasitoid attack.

To maximize survival, invertebrate animals face trade-offs between food quality and predator avoidance (Singer *et al.* 2004b, Kumagai 2008). The value of enemy-free space can, in many instances, surpass that of food quality, and selection has promoted host-plant shifts among insect herbivores in favour of predator avoidance (Murphy 2004). Here, refuge from natural enemies may be provided by a different part of the same host plant, *i.e* the flowers. Flowers are good food for the herbivores and provide refuge from their specialist parasitoid. *Cotesia glomerata* mostly attack young caterpillars on leaves rather than on flowers of *B. nigra* plants. We expect that the benefit of flowers as high-quality food and escape from the specialist parasitoid select for flower feeding in *P. brassicae*. Selection may favour the choice of adult herbivores for plants in the flowering stage, and for second-instar larvae to move to flowers, but seems to operate differently at the later stages of larval development. Caterpillars were highly preyed upon when feeding on the flowers.

Herbivores in nature are exposed to multiple enemies, and different larval stages are preferred by different attackers (Clancy & Price 1987, Williams 1999). Behavioural responses to natural enemies may have trade-offs because avoiding one enemy might mean running into another (Denno *et al.* 2003). In this study, larger caterpillars of *P. brassicae* that fed on flowers were more vulnerable to predation by social wasps than small young larvae that fed on leaves. *Polistes dominula* and *Vespula* sp. were the main predators of the late instars of *P. brassicae*. Overall, 42 % and 67 % of late-instar caterpillars were killed by these social wasps in the two trials of these experiments. High predation rates of late-instar *P. brassicae* caterpillars were also observed in our previous study; overall mortality by predation was 88 % and 43 % in two trials, during the period in which caterpillars were feeding on the flowers (Lucas-Barbosa *et al.* 2013). These wasps are generalist predators that consume a broad range of prey, including pierid caterpillars (Richter 2000, Picanco *et al.* 2011). Interestingly, social wasps were only observed at the end of each trial of our field experiments, *i.e* when third to fifth instar larvae were feeding on flowers of *B. nigra* plants. These wasps were not observed visiting our plots when plants had only eggs or early-stage caterpillars that fed from leaves. Social wasps are known to use both visual and olfactory cues, including herbivore-induced plant volatiles, when foraging for prey (Richter 2000, Brodmann *et al.* 2008). From a long distance, olfactory cues may attract social wasps to *B. nigra* plots with plants infested with large prey. Odours of *B. nigra* plants indeed change throughout the flowering stage (Lucas-Barbosa *et al.* 2013) and this might explain why social wasps were specifically attracted to plots of *B. nigra* plants when those carried late-instar caterpillars. In our study, *P. dominula* and *Vespula sp.* spent more time searching for caterpillars in caterpillar-infested plots than in non-infested plots. At short distances, social wasp foraging might also be based on visual cues. When we tested whether feeding site of the caterpillars would affect foraging efficiency of social wasps, we placed caterpillars on either leaves or flowers of plants and found that mortality on these sites was similar. This suggests that social wasps exploit cues that are associated with plants infested with rewarding prey, such as large caterpillars of the gregarious *P. brassicae*, but at a short distance can find caterpillars on either flowers or leaves of *B. nigra* plants. Thus, we do not expect that caterpillars could increase their survival chances by returning to leaves at a later stage of their development, when they are no longer susceptible to parasitism by *C. glomerata*. Flower odours may, therefore, influence long-range attraction of social wasps to plants infested with large caterpillars, but at a short range these wasps might also exploit visual cues, and caterpillars are at risk of attack on leaves and flowers of *B. nigra* plants.

Flowers of *B. nigra* plants may still be the best food choice for *P. brassicae* caterpillars because larvae develop faster when feeding on flowers, and may benefit from flower feeding if they can reach the reproductive stage and produce offspring (Benrey & Denno 1997). Generalist carnivores are expected to maintain continuous pressure on herbivores, and on their food-plant selection (Bernays 1988). *Pieris brassicae* is a gregarious species and feeds on *B. nigra* that is rich in glucosinolates (Smallegange *et al.* 2007). Social wasps deal with the high content of defensive chemistry by removing the gut of *P. brassicae* prey (Rayor *et al.* 2007). Gut removal is an advantageous tactic (Rayor *et al.* 2007) and may allow these wasps to prey on a wide range of insect species and succeed as generalist foragers. Gregariousness might render *P. brassicae* caterpillars especially attractive to social wasps. Adult butterflies lay up to 100 eggs on a single brassicaceous plant. The first two instars feed gregariously and later instars disperse to the neighbouring plants through the inflorescences of *B. nigra* plants (Lucas-Barbosa *et al.* 2013). For social wasps this means that a large number of prey can be found in a relatively small area. These generalist foragers may benefit from finding species that feed gregariously because these wasps can learn and precisely return to a profitable site (D'Adamo & Lozada 2008), whereas for each individual herbivore the chances of being killed may be diluted (Wertheim *et al.* 2005).

This study shows that flowers influence host-plant selection by adult herbivores, and subsequent parasitoid-host and predator-prey interactions. Flowers are a refuge from parasitoids for the caterpillars. However, florivorous larvae can be intensively preyed upon by social wasps. Yet, because the caterpillars develop faster on flowers than on leaves (Smallegange *et al.* 2007), flower feeding may be still advantageous if this enhances the chances to reach the reproductive stage. Flower preference by *P. brassicae* is more likely driven by the nutritional benefit and reduced risk of parasitism by the specialist endoparasitoid on flowers, than by risks of being caught by generalist predators.

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Chapter 7

Seed set of mustard plants is compromised in the absence of natural enemies of herbivores

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Abstract

Plants are under natural selection to maximize their reproductive success. When regarding reproduction, most flowering plants rely on insect pollinators. However, to be able to reproduce plants need to continuously defend themselves against insect herbivores. Thus, to maximize fitness, plants must balance between investments in constitutive and inducible response mechanisms against herbivores and investments in reproduction. Plants can resist to herbivory *via* phytochemical responses that directly affect the herbivores, or that increase effectiveness of carnivorous insects – so called indirect resistance. To compensate for fitness costs due to herbivory, plants can allocate resources to regrowth of vegetative tissues or to reproductive tissues. Here, we investigated how resources are allocated by flowering *Brassica nigra* plants upon attack by *Pieris brassicae* caterpillars, and studied compensatory mechanisms in which plants invest to maintain fitness along with the indirect resistance. The data show that plants respond to caterpillar feeding by allocating resources to reproduction and can compensate for fitness loss due to herbivory in terms of seed production when interactions with carnivores are maintained. However, in the absence of carnivores, *B. nigra* could not compensate for the damaged caused by *P. brassicae* caterpillars. Our data indicate that the interaction with natural enemies of herbivores is determinant in maintaining fitness of *B. nigra* plants.

Keywords: compensation, indirect defence, night pollinators, plant fitness, plant volatiles, resource allocation.

Introduction

Plants are among the most speciose groups of organisms on Earth and are involved in intricate interactions with herbivores, carnivores and pollinators (Schoonhoven *et al.* 2005). Rooted in soil, plants have evolved multiple resistance mechanisms against insect herbivores and mechanisms to compensate for the damage caused by them while maintaining interactions with pollinating insects, and ultimately maximizing plant fitness (Dicke & Hilker 2003, Agrawal 2011, Fornoni 2011).

To reduce or avoid damage, plants produce from a few to several hundreds of metabolites that provide constitutive plant resistance (Schoonhoven *et al.* 2005, Agrawal 2011). This baseline of phytochemical resistance present in plants is, as a blend, species-specific and protects plants from being eaten by most insects (Schoonhoven *et al.* 2005). In addition, plants can also invest in the production of secondary metabolites in response to herbivory (Kessler & Baldwin 2002, Dicke & Baldwin 2010). Production of plant secondary metabolites induced by damage may minimize biosynthetic costs and confers plasticity to this direct form of resistance to herbivores (Karban & Baldwin 1997, Nishida 2002, Hopkins *et al.* 2009). Induced direct resistance can affect herbivores above and belowground (Soler *et al.* 2007, Erb *et al.* 2008, Howe & Jander 2008, Erb *et al.* 2009, Mithofer & Boland 2012), and such responses might differ depending on the feeding guild of the herbivores (Stam *et al.* 2014). Among the phytochemical responses induced by herbivory, plants can invest in the production of volatile compounds. The production of herbivore-induced plant volatiles (HIPVs) that are exploited by predators and parasitoids of herbivores is regarded as indirect plant resistance. Plants can respond to herbivory with changes in volatiles emitted by leaves and flowers, and in this way attract the natural enemies of its own enemies (Kessler & Baldwin 2001, Dannon *et al.* 2010, Mumm & Dicke 2010, Lucas-Barbosa *et al.* 2011, Pareja *et al.* 2012, Bruinsma *et al.* 2014).

Plants do not only respond to limit herbivore damage, but they can also respond in other ways in order to minimize the effect of herbivory on plant fitness (Fornoni 2011). Such responses include increased photosynthetic activity, an increase in nutrient uptake, and reallocation of resources; those responses can result in compensation of tissue loss due to herbivory (Rosenthal & Kotanen 1994, Strauss & Agrawal 1999, Strauss & Murch 2004, Schwachtje & Baldwin 2008, Fornoni 2011). Plants can also reduce fitness costs of herbivory by allocating resources specifically to reproduction (Penet *et al.* 2009, Lucas-Barbosa *et al.* 2013, Pashalidou *et al.* 2013). In fact, herbivory is known to influence plant reproduction in a number of ways, for example by affecting pollinator behaviour, speed of reproduction, and selfing rates (Poveda *et al.* 2003, 2005, Kessler & Halitschke 2009, Penet *et al.* 2009, Lucas-Barbosa *et al.* 2011, Lucas-Barbosa *et al.* 2013, Pashalidou *et al.* 2013). Herbivory can lead to an increased attraction of pollinators, but can also negatively affect their behaviour (Poveda *et al.* 2003, 2005, Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011). Results of our recent study showed that Black mustard plants, *Brassica nigra,* respond to egg deposition by the Large Cabbage White butterfly *Pieris brassicae* by accelerating seed production (Lucas-

Barbosa *et al.* 2013), which is advantageous for these plants because the larvae feed on flowers, but not on the seeds. Thus, plants can safeguard reproductive output by speeding up reproduction (Lucas-Barbosa *et al.* 2013). Selfing is a strategy through which plants can ensure reproduction in the absence or under limiting visitation rates by pollinators, and this can be increased upon herbivory (Harder & Barrett 2006, Penet *et al.* 2009). Thus, plants can invest in compensatory mechanisms to ensure reproduction after herbivore attack.

The extent to which plants can invest in induced resistance and in compensatory mechanisms in response to herbivore attack is key in maximizing fitness (Núñez-Farfán *et al.* 2007), and may vary with plant ontogeny (Diezel *et al.* 2011, Quintero & Bowers 2011). For instance, juvenile stages of *Penstemon virgatus* compensated for herbivory in terms of plant biomass production, whereas young seedlings showed no compensatory re-growth. However, the capacity of induced responses to reduce herbivore damage, *i.e.* induced resistance, was conserved throughout the development of this perennial herb (Quintero & Bowers 2011). In the foliage of the solanaceous plant *Nicotiana attenuata*, inducibility of phytohormones in response to herbivory was attenuated as the plant developed from the vegetative to the generative stage. Consequently, herbivory did not induce resistance in leaves of flowering *N. attenuata* (Diezel *et al.* 2011). However, this study did not quantify whether herbivory elicited the production of phytohormones in flower tissues. In fact, induced resistance can be attenuated in foliage (Diezel *et al.* 2011), and increased in flowers during the reproductive stage (Bruinsma *et al.* 2014). Therefore, investments in induced resistance and compensatory mechanisms can vary during plant development and allocated to the most valuable tissues for a given plant, and at a given point in time (McKey 1974).

Upon herbivore attack, plants respond in multiple ways that include compensatory mechanisms, induced direct resistance against herbivores, and enhanced interactions with mutualistic insects (Poveda *et al.* 2003, Strauss & Murch 2004, Schwachtje & Baldwin 2008, Dicke & Baldwin 2010, Agrawal 2011). To understand the ecological and evolutionary significance of these multiple strategies, it is crucial to investigate how responses induced by herbivores can be translated into plant fitness. In a combination of field and greenhouse studies, we here explored how *B. nigra* plants balance investments between reproduction and defensive strategies. The ultimate goal was to investigate how *B. nigra* compensates for herbivory by *P. brassicae* caterpillars. In the field, despite the extensive damage caused by *P. brassicae* to vegetative and reproductive tissues, mustard plants can compensate for herbivory, producing as many or even more seeds than non-infested plants (Lucas-Barbosa *et al.* 2013). Maintenance of interactions with mutualistic insects seems important in this system. Carnivorous insects can significantly reduce herbivore abundance on *B. nigra* leading to mortality of *P. brassicae* that can get as high as to 95%, according to data collected in two consecutive years of field experiments (Lucas-Barbosa *et al.* 2013, Lucas-Barbosa *et al.* 2014). Under herbivore attack, mustard plants maintain interactions with pollinating insects, but compensation cannot be explained by enhanced attraction of day-active pollinators to herbivore-infested plants (Lucas-Barbosa *et al.* 2013). Here, the underlying mechanisms of compensation in *B. nigra* were addressed by asking the following questions: 1) How do *B. nigra* plants allocate resources upon herbivore infestation by *P. brassicae*? 2) Can *B. nigra* be self-fertilized and does herbivory increase self-fertilization? 3) Is reproduction in *B. nigra* mainly mediated by day- or night-active pollinators? 4) Can *B. nigra* compensate for herbivory in terms of seed production when predators and parasitoids of *P. brassicae* are absent?

Materials and methods

Study system

Brassica nigra L. (Brassicales: Brassicaceae) is an annual plant that is considered to be obligately outcrossing; in nature it is pollinated by various insects including bees, syrphid flies and butterflies (Conner & Neumeier 1995, Lucas-Barbosa *et al.* 2013). In The Netherlands, this wild species occurs as an early successional plant and stands in high densities.

Pieris brassicae L. (Lepidoptera: Pieridae) is a specialist herbivore of plants in the family Brassicaceae. This lepidopteran species is gregarious and female butterflies lay clutches of up to 100 eggs, generally on the underside of leaves. When larvae hatch from the eggs, caterpillars initially feed gregariously on leaves of a flowering *B. nigra* plant, but second-instar larvae soon move to flowers. In The Netherlands, *P. brassicae* caterpillars are frequently attacked by various carnivorous insects: 1st and 2nd instar larvae can be attacked by the gregarious parasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae). Young larvae can also be preyed upon by ladybird beetles, and later instars can be intensively preyed upon by social wasps (Lucas-Barbosa *et al.* 2013, Lucas-Barbosa *et al.* 2014).

Episyrphus balteatus (De Geer) (Diptera: Syrphidae) is one of the most common syrphid fly species worldwide (Jauker & Wolters 2008). Larvae of this syrphid fly feed on aphids, and, therefore, syrphid flies have been widely used as biological control agents. Adult syrphid flies feed on nectar and pollen, but collect mainly pollen from *B. nigra* flowers (D. Lucas-Barbosa, personal observation). *Episyrphus balteatus* adults serve as pollinators, increasing the fitness of *Brassica* plants (Jauker & Wolters 2008).

plants and insects

Seeds of an early-flowering accession (CGN06619) of *B. nigra* were obtained from the Centre for Genetic Resources (CGN, Wageningen, The Netherlands) and multiplied by exposing plants to open pollination in the surroundings of Wageningen. Seeds collected from 25 plants were mixed to obtain seed batches for the experimental plants. For the greenhouse experiments, potted *B. nigra* plants were reared in a greenhouse compartment (23 ± 2 °C, 50-70% r.h., L16:D8). For the field experiments, *B. nigra* potted plants were reared outside on tables protected by insect screens, in a location close to the field site. Plants in the flowering stage, with several flowers open, and in the vegetative growth stage, *i.e.* stages 4.2 and 3.2 respectively, based on classification for *Brassica napus* (Harper & Berkenkamp 1975), were used for the experiments. *Pieris brassicae* were obtained from a laboratory colony reared on Brussels sprouts (*Brassica oleracea* L. var. *gemmifera*) plants in a climate room (22 ± 1 °C, 50-70% r.h., L16:D8). The adults were provided with a 10% sucrose solution as food. *Episyrphus balteatus* pupae were obtained from Koppert B.V., Berkel en Rodenrijs, The Netherlands. Adult flies were kept (22 ± 1 °C, 50-70% r.h., L16:D8) in a cage with a Brussels sprouts plant infested with the cabbage aphid *Brevicoryne brassicae*, known to promote the development of the female reproductive system (M. Kos, personal communication). Adult syrphid flies had access to sugar, pollen and water.

Plant treatment

Flowering plants with a few open flowers were infested with one egg clutch of *P. brassicae* by exposing plants to butterflies. For the greenhouse experiments, plants were exposed to *P. brassicae* butterflies in an oviposition cage (100 cm × 70 cm × 82 cm). While the plant was inside the oviposition cage, flowers were covered with a mesh bag to prevent being visited by the butterflies. For the field experiments, we exposed each plant to a mated female butterfly while the plant was covered with a tent. In field and greenhouse conditions, the number of eggs on a plant was reduced to 30 by gently removing surplus eggs shortly after egg deposition by the butterfly had been interrupted.

Resource allocation by *Brassica nigra* upon herbivore infestation

We estimated how resources are allocated between leaves and reproductive tissues of *B. nigra* plants upon herbivory. At three time points, we measured total nitrogen and organic carbon in leaf and flower tissues of infested plants and non-infested control plants at the same development stages, and used this as a proxy of resource allocation. To do this, infested and control plants were kept in a greenhouse compartment (23 ± 2 °C, 50-70% r.h., L16:D8) until leaves and flowers were harvested. Carbon and nitrogen contents were determined at three time points: 1) egg-infested plants (4 days after oviposition), 2) leaf-infested plants (folivory - 1 day after caterpillars had hatched from the eggs) and 3) flower-infested plants (florivory - 5 days after caterpillars had hatched from the eggs).

At these three time points, leaves and flowers of control and infested plants were harvested, frozen in liquid nitrogen and freeze-dried. All herbivores were removed from the plants before freezing the samples. Dried samples were ground, weighed and stored at −20 °C. Total nitrogen and organic carbon in leaf and flower tissues of the infested plants were then determined by dry combustion using a CHN analyser and compared with that of non-infested control plants at the same development stages. Data were analysed using a generalized linear model and the Student’s t-test.

Selfing in *Brassica nigra* plants upon herbivore infestation

We tested if *B. nigra* increased selfing rates when exposed to herbivore infestation. To do this, *B. nigra* plants were transplanted to the field at bud stage and individually isolated in tents (95 x 95 x 190 cm, white fine polyester mesh). Once the plants were flowering, 50% of the plants used for the experiments were infested with one egg clutch of *P. brassicae* and 50% were kept as control plants. After the larvae had hatched, the caterpillars were allowed to feed on the plant for 48 h, and then removed. On the day when plants were infested with eggs, two adult syrphid flies and two male *P. brassicae* butterflies were released in each of the tents containing an individual *B. nigra* plant so that pollen could be carried among flowers of the same plant. Adult syrphid flies and butterflies were replaced every week until the plants were harvested. Plants remained in the field in tents isolated from other plants for 27 days. Once harvested, aboveground dry plant biomass and seed biomass produced by infested and control plants were determined. Plant shoots were dried overnight (105 °C), and dry biomass was measured. When the total number of seeds was smaller than one hundred for an individual plant, all seeds were counted. When the number of seeds was larger than one hundred seeds, the total number of seeds was estimated by dividing the total seed weight by the weight of one hundred seeds of that plant. In total 60 plants were used for these experiments. Data were analysed using a generalized linear model.

Visitation of *Brassica nigra* flowers by day- and night-active pollinators and Plant volatile emission during day and night

Pollinator visitation to *B. nigra* flowers was recorded during the day and the night, under field conditions. During the day, pollinator visitations were recorded by direct observation for 10 min using a handheld computer (Psion Workabout) programmed with The Observer XT software (version 10, Noldus Information Technology, Wageningen, The Netherlands). Observations were carried out whenever weather conditions were suitable (17-25 °C – wind speed ≤ 6 m s^{-1}), between 9 am and 4 pm (details outlined by Lucas-Barbosa et al. (2013)). During the night, infrared cameras were used to record flower visitation by night-active insects. Each night, visitation to one or two plots of plants was recorded with an infrared camera and infrared light. During nine different nights, different plots, each with five plants in the flowering stage, were observed with the infrared camera for a total of 36 h. Images were captured using two analogue monochrome cameras (Ikegami, ICD-49E) at 5 frames per second. The near infra-red cut filter was removed from the camera to make it sensitive to near infrared [see Allema *et al.* (2012) for more details]. Radiation sources were placed next to each of the cameras. The cameras were connected to a laptop and StreamPix software (4 x 64 edition, Norpix Inc., Montreal, Canada) was used for recording the video files (in AVI-format). Observations were carried out whenever weather conditions were suitable (10 – 30 °C – wind speed ≤ 8 m sec^{-1}, and no rain), between 8 pm and 8 am. Eight videos of 30 min were recorded per night. Field layout consisted of 16 plots of *B. nigra* plants and each plot (50 × 50 cm) was composed of 5 plants (details have been previously described by Lucas-Barbosa *et al.* (2014)). The observations were carried out between July and August 2012, at the same site as earlier experiments, *i.e.* in an experimental field site in Wageningen, The Netherlands. Data were analysed using the Mann-Whitney U test.

Plant volatiles were collected from aerial parts of *B. nigra* plants during day and night. Experiments were performed in a greenhouse compartment (22 ± 2 °C, 60 ± 10% r.h. L16:D8) and headspace volatiles were collected for 1.5 h. Day collection was performed between 10 am and 3 pm and night collection was performed 1 h after the scotophase had started. Aerial parts of plants were enclosed in an oven bag (Toppits® Brat-Schlauch, polyester; 32 x 32 x 70 cm; Toppits, Minden, Germany). A strip of bag material wrapped around the stem and above the inflorescence was used to close the bag. Synthetic air was flushed through the bag at a flow rate of 300 mL min^{-1} by inserting Teflon tubing through an opening in the upper part of the bag. Air was sucked out (224-PCMTX8, air-sampling pump Deluxe, Dorset, UK; equipped with an inlet protection filter) from the bag enclosing the aerial parts of the plants, and headspace volatiles were collected on Tenax (90 mg of Tenax-TA 25/30 mesh, Grace-Alltech) in a glass tube at a flow rate of 250 mL min^{-1}. Bags were discarded after use. Volatiles from 11 individual plants were collected. Headspace samples were then analysed in a gas chromatograph with a thermodesorption unit (GC) (6890 series, Agilent, Santa Clara, USA) connected to a mass spectrometer (MS) (5973 series, Agilent, Santa Clara, USA). We followed the methods outlined by Bruinsma *et al.* (2014) to analyse, identify and quantify volatiles emitted by *B. nigra* plants. Projection to Latent Structures Discriminant Analysis (PLS-DA) (SIMCA P+ 12.0, Umetrics AB, Umeå, Sweden) was used to determine whether the samples subjected to the different treatments could be separated based on the composition of the volatile blend. To determine significant differences between emission of volatile compounds by *B. nigra* plants during daytime and night time we used Wilcoxon matched-pairs tests as assumptions of normality were not met.

SEED PRODUCTION UPON HERBIVORE INFESTATION IN THE PRESENCE AND ABSENCE OF NATURAL ENEMIES

We investigated whether *B. nigra* plants can compensate for herbivory by *P. brassicae* in comparison with control plants. Compensation for herbivory in terms of plant biomass and seed production was tested in two field conditions: 1) in open field conditions where plants and introduced herbivores were exposed to naturally occurring insects, including pollinators and carnivorous insects; 2) in conditions where natural enemies (carnivorous insects) were excluded, and syrphid flies (*E. balteatus*) and male butterflies (*P. brassicae*) were used to ensure cross-pollination between plants of the same plot. For each of these two conditions, the field layout consisted of 16 plots of *B. nigra* plants infested with *P. brassicae* and 16 control plots. Each plot (50 × 50 cm) was composed of five plants. The central plant of the plot was either infested with 30 *P. brassicae* eggs or was non-infested. The other four plants were all non-infested. Plots with an infested central plant are termed “infested plots” and plots with a non-infested central plant are termed “control plots”. Equal numbers of control and infested plots were transplanted to the field on eight consecutive days. Plants that were infested on the same day were never planted in the same column or row in the field lay-out. Control and infested plots were planted alternately, and the distance between them was 1.5 m. When carnivorous insects were excluded, a tent was used to cover each plot of plants (95 x 95 x 190 cm, white fine polyester mesh) and two adult syrphid flies and two male *P. brassicae* butterflies were released in each of the tents as pollinators, within one week after eclosion from pupae. Adult syrphid flies and butterflies were replaced every week until the plants were harvested. Once harvested, aboveground dry plant biomass and seed biomass produced by infested and control plants in the two given conditions were determined as described above. We also measured the biomass of the herbivores reared in these two conditions. Caterpillars were recollected and weighed 28 days after egg deposition by the butterfly, for both the open field and exclusion conditions. We carried out two serially repeated trials between May and July 2013, at an experimental field site in Wageningen, The Netherlands. Data were analysed using a generalized linear model.

Results

Resource allocation by *Brassica nigra* upon herbivore infestation

Overall, C/N ratios decreased upon herbivore infestation of flower tissues (Fig. 1, GLM, *P* = 0.037), but not in leaf tissues (Fig. 1, GLM, *P* = 0.117) compared to C/N ratios in flowers and leaves of control plants. Initial feeding damage by caterpillars, however, resulted in remarkable changes in C/N ratio in leaf tissues (Fig. 1e, Student’s t-test, $P \leq 0.001$) apart from the changes observed in flower tissues (Fig. 1d, Student’s t-test, *P* = 0.019). The observed changes in C/N ratios generally resulted from changes in nitrogen content rather than changes in carbon content. For instance, folivory induced changes in nitrogen content of leaves (Student’s t-test, *P* = 0.010) and flowers (Student’s t-test, *P* = 0.038), but did not induce changes in the carbon content of leaves (Student’s t-test, *P* = 0.593) or flowers (Student’s t-test, *P* = 0.485).

Selfing in *Brassica nigra* plants upon herbivore infestation

Plants induced by *P. brassicae* feeding produced as many selfed seeds as control plants (GLM, *P* = 0.249), and reached similar aboveground plant biomass (GLM, *P* = 0.913). We observed an effect of the trial on seed production (GLM, $P \leq 0.001$) and plant biomass (GLM, $P \leq 0.001$), but no overall effects of the treatment, and no interaction between treatment and trial (GLM, *P* = 0.102, *P* = 0.166).

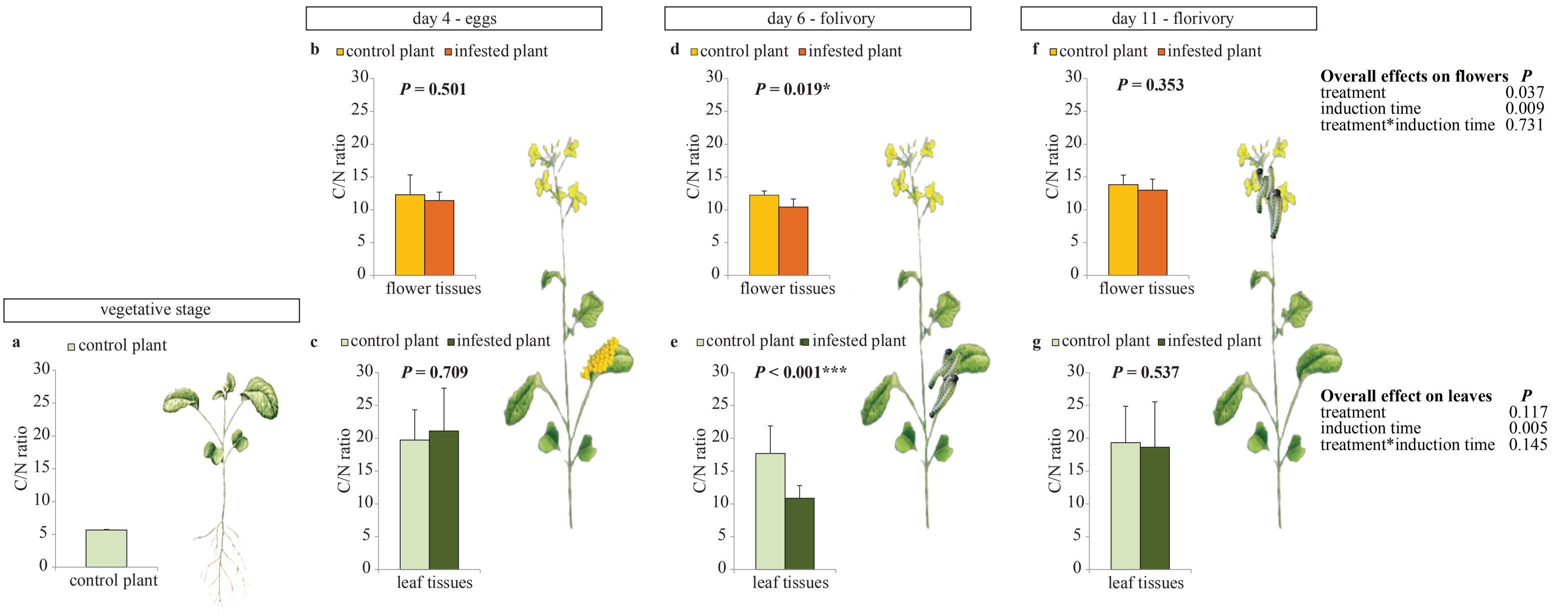

Fig. 1 Carbon-nitrogen (C/N) ratios of *Pieris brassicae*-infested and (non-infested) control *Brassica nigra* plants. C/N ratio (mean ± SD) is shown for control plants in the vegetative stage (a) and for leaf and flower tissues of plants infested with eggs (b, c), and caterpillars that feed on leaves (d, e) and flowers (f, g) along with control plants of equivalent development stage. Plants were infested with *P. brassicae* eggs on the day the first flowers were open. Five plants per treatment/development stage were used. A generalized linear model and the Student's t-test were used to determine significant differences at 0.05 level.

Visitation of *Brassica nigra* flowers by day- and night-active pollinators and Plant volatile emission during day and night

Brassica nigra flowers were rarely visited by insects during the night. Thus visitation rates during the day were significantly higher than during the night (Fig. 2, Mann-Whitney U, $P \leq 0.001$). During daytime, the pollinator species most frequently observed was the honey bee, *Apis mellifera* L. (Hymenoptera: Apidae). Many species of syrphid flies were also abundant flower visitors, including the syrphid fly *Eristalis tenax* (Diptera: Syrphidae). Diptera: Syrphidae). Solitary bees, butterflies and bumblebees were also observed visiting *B. nigra* flowers, but in lower numbers in comparison with honey bees and syrphid flies. During night time, a total of 11 moths were observed flying through the plots of *B. nigra* plants during the nine nights we recorded visitation. Only four moths were actually observed visiting the flowers. Of these four individual moths, three moths were identified as a member of the Noctuidae family and they visited 8 flowers altogether. The fourth moth was a microlepidopteran which visited one flower. The total time spent by all moths on the flowers represented less than 1% of the total recording time.

Volatile emission rates by *B. nigra* plants were considerably lower during night time when compared with emission rates during daytime (Fig. 2, Wilcoxon matched pairs tests, $P \leq 0.001$). A Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile compounds collected from flowering *B. nigra* plants during the day and night resulted in a model with 2 principal components; the first principal component explained 55% and the second principal component 9 % of the total variance (see supporting information in Appendix E, Fig. E1). The PLS-DA completely separated the volatile profile of plants collected during the day from that of night collection (Fig. E1). During daytime, several compounds were emitted in larger amounts than during the night (Appendix E, Table E1).

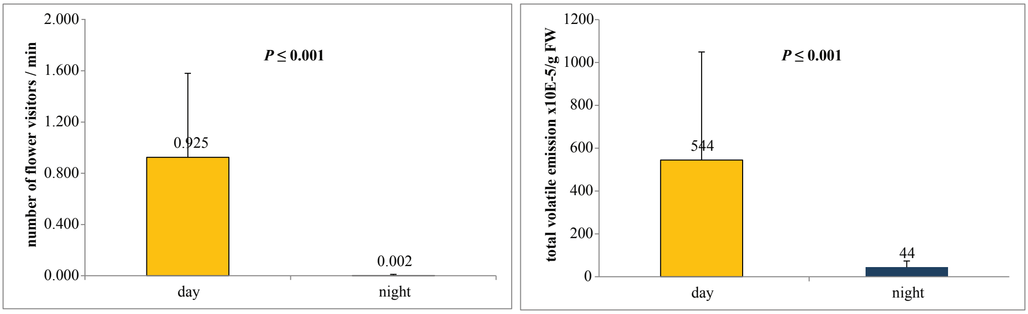

Fig. 2 Number of flower visitors of *Brassica nigra* (a) and total volatile emission (peak area) (b) by these plants during day and night. Number of flower visitors (mean + SD) per minute is shown. Values for peak area (mean ± SD) obtained from total ion chromatogram were divided by 10^5 and expressed per gram of plant fresh weight (FW). Mann-Whitney U and Wilcoxon matched-pairs tests were used to determine significant differences at the 0.05 level.

SEED PRODUCTION UPON HERBIVORE INFESTATION IN THE PRESENCE AND ABSENCE OF NATURAL ENEMIES

In open field conditions where carnivorous insects had access to the plants, infested and control plants produced similar numbers of seeds: both for the plants originally infested with butterfly eggs (Fig. 3a, A-plants, GLM, $P = 0.988$), and for all plants within the plot (Fig. 3b, GLM, $P = 0.381$). In conditions where carnivorous insects and pollinators were excluded, infested plants produced fewer seeds than control plants. Effects of herbivore treatment were observed for the plants originally infested with eggs (Fig. 3c, A-plants, GLM, $P = 0.005$), and at plot level (Fig. 3d, GLM, $P = 0.006$). To estimate re-growth capability in *B. nigra*, aboveground biomass was determined for infested and control plants. Shoot dry weights of infested and control plants were generally similar for experiments carried out in the two conditions, but there was an effect of trial. Irrespective of the treatment, plants of trial 2 produced lower biomass than plants of trial 1 (Appendix E, Fig. E2).

In open field conditions, we recorded 46% survival of the caterpillars, whereas in the conditions where carnivores were excluded, 80% of caterpillars were recollected at the end of trial 1. It is interesting to note that caterpillars under conditions where natural enemies of the herbivores were excluded were heavier than those under conditions where natural enemies were present (Appendix E, Fig. E3, Student's t-test, $P \leq 0.001$). No caterpillars were recovered for trial 2 in open field conditions.

Discussion

Brassica nigra plants allocated resources to reproductive tissues. This plant species compensated for herbivory when exposed to naturally occurring insects, including natural enemies of the herbivores. Seed production by *B. nigra* plants was, however, compromised in the absence of those natural enemies. Our results suggest that multiple response mechanisms, *i.e.* reallocation of resources and the interaction with carnivorous insects, both contributed to sustaining fitness of *B. nigra* plants.

Brassica nigra plants reallocated resources from leaves to reproductive tissues upon the deposition of eggs by *P. brassicae* butterflies. Overall, C/N ratios declined upon herbivore infestation in flower tissues. Initial feeding damage by caterpillars, however, resulted in a significant decline in C/N ratio in both leaf and flower tissues, which may imply that plants increase nitrogen uptake once caterpillars start to feed. In fact, changes in C/N ratios generally resulted from increases in nitrogen concentration rather than changes in carbon concentrations. Evidence from physiological studies indeed suggests that carbohydrates are often not enough or cannot be at all mobilized upon herbivory, but plants may continue to depend upon reserves of nitrogen even after deployment of carbon storage has declined (Rosenthal & Kotanen 1994, Strauss & Agrawal 1999). This post-damage allocation pattern

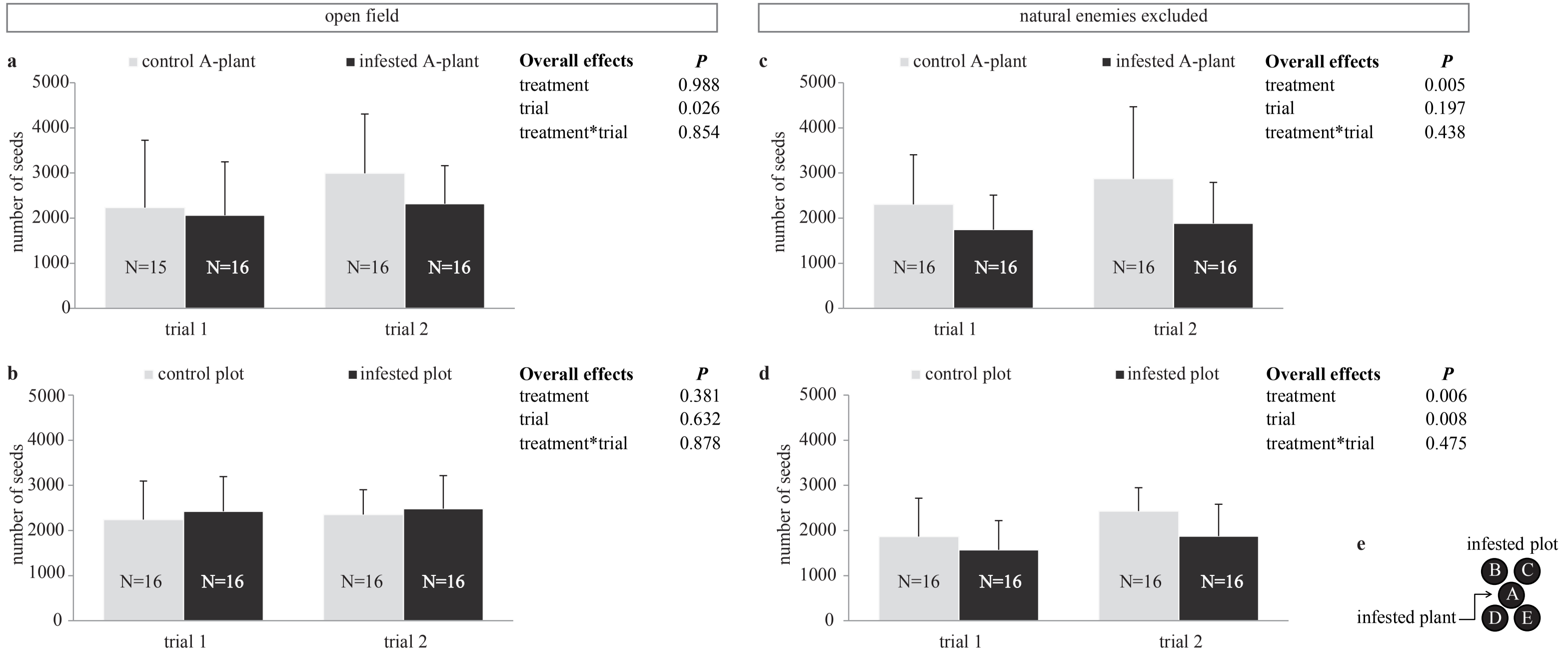

Fig. 3 Number of seeds produced (mean + SD) by *Pieris brassicae*-infested and control *Brassica nigra* plants in open field and when natural enemies of *P. brassicae* were excluded. In open field, plants and herbivores were exposed to naturally occurring pollinators and carnivorous insects. In the condition where natural enemies (carnivorous insects) were excluded, syrphid flies (*Episyrphus balteatus*) and male butterflies (*P. brassicae)* were used to ensure cross-pollination between plants of the same plot. Data are shown for the central plant (A-plant) of the plot (a,c) and at plot levels (b,d) for trials 1 and 2. Schematic representation of a field plot (e); each plot is composed of 5 plants, and the central plant of the plot was either infested with 30 *P. brassicae* eggs or was non-infested; second through fifth instar larvae colonized the other four plants of infested plots. A generalized linear model was used to determine significant differences at $\alpha = 0.05$. Number of plants and plots is shown in the bars.

reveals that plants invest in reproductive tissues.

Early investments in reproduction can contribute to compensation in terms of seed production. Our previous studies show that *B. nigra* plants accelerated flower (Pashalidou *et al.* 2013) and seed production (Lucas-Barbosa *et al.* 2013) in response to butterfly egg deposition, and then compensated for herbivory in terms of plant biomass and seed production (Lucas-Barbosa *et al.* 2013). Infested *B. nigra* plants were as attractive as control plants to day-active pollinators, in this field experiment (Lucas-Barbosa *et al.* 2013). Here, we aimed to investigate the underlying mechanisms. We wanted to understand how *B. nigra* compensates for herbivory and considered whether flowers were visited during the night. This could possibly lead to more successful pollination of herbivore-infested plants if herbivore induction could render plants more attractive to night-active pollinators. We investigated also whether flowers of *B. nigra* could be self-fertilized, and whether herbivory would lead to an increase in selfing rates. Our data show that although the yellow flowers of *B. nigra* remain open during the night, these flowers are rarely visited by night-active insects when compared with visitation rates during the day. This correlates well with the fact that volatile emission is largely reduced when it is dark, rendering plants probably less attractive to night-active pollinators. Moths are generally attracted to white, pale and yellow flowers (Miller *et al.*, 2011). However, moths exploit colours as short range cues, whereas odour cues play a more important role in long-distance orientation (Kevan & Baker, 1983). When regarding *B. nigra* flowers, it is likely that moths cannot detect relatively low odour production by inflorescences from a long distance, and are, therefore, not attracted to the plants from long-range during the night. *B. nigra* flowers are visited by butterflies during the day, although at much lower rates when compared with visitation by bees and syrphid flies (Lucas-Barbosa *et al.* 2013). Day-active pollinators are more likely to contribute to seed set of *B. nigra* plants than night-active pollinators.

In this study, we discovered that *B. nigra* can be self-fertilized, although this species had to date been considered to be an obligately outcrossing species. We would estimate that roughly about 40% of the seeds are selfed, if we compare the number of seeds produced by outcrossed and selfed plants. Selfing rates in our experiments were, however, not influenced by prior infestation by *P. brassicae*. Herbivory can influence plant reproduction in a number of ways, including an increase in selfing rate as a result of florivory (Penet *et al.* 2009), and floral sex-ratio can be affected by simulated weevil herbivory (Wise & Hebert 2010). In this study, selfing does not explain how *B. nigra* compensated for herbivory in terms of seed production. Self-incompatibility in plants is, in many cases, promoted by an unequal ripening of the male and female organs (dichogamy), which leads to female parts maturing too late to be fertilised by pollen from the same plant, which is already dispersed (Lloyd and Webb, 1986). We suggest that herbivory may lead to an accelerated development of the female reproductive organs in *B. nigra*, but reproduction seems more successful when pollen comes from a different plant. Thus, although roughly about 40% of the seeds are selfed in our experiments, we would like to highlight that *B. nigra* benefits when pollen is transferred from one plant to another, as outcrossed plants are likely to produce more seeds than selfed plants. Yet, selfing and the accelerated reproduction in response to herbivory represent different strategies through which *B. nigra* plants ensure reproduction.

Upon herbivore infestation, *B. nigra* plants reallocate resources (this study) and the production of plant secondary metabolites (Bruinsma *et al.* 2014) into reproductive tissues, and can compensate for herbivory when interactions with pollinating (Lucas-Barbosa *et al.* 2013, Bruinsma *et al.* 2014) and carnivorous insects are preserved. Indeed, the present study shows that in the absence of natural enemies of *P. brassicae,* fitness of *B. nigra* plants was negatively affected. Although infested plants could still regrow and compensate in terms of vegetative biomass, plants could not compensate in terms of seed production. *Pieris brassicae* is a voracious feeder, and 2nd through 5th instar larvae feed exclusively on the flowers of *B. nigra* plants, entirely consuming large numbers (Smallegange *et al.* 2007, Lucas-Barbosa *et al.* 2013). This study indicates that carnivorous insects are essential in maintaining herbivore pressure low.

In sum, our results indicate that the interaction with natural enemies is vital in maintaining herbivore pressure in *B. nigra* low. Plants respond to herbivory with multiple inducible traits, and for *B. nigra* compensatory mechanisms and the attraction of natural enemies can contribute to fitness. However, when carnivores were absent, plants suffered from herbivory producing fewer seeds than control plants. We conclude that carnivores play an important role in maintaining herbivore pressure low on this short-lived brassicaceous plant.

Acknowledgements

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Chapter 8

General discussion: integrating plant-pollinator and plant-herbivore interactions

Dani Lucas-Barbosa

Plants and insects are among the most abundant groups of organisms on Earth (Schoonhoven *et al.* 2005). Biodiversity is higher among angiosperms than in any other group of plants (Frame 2003, Harder & Barrett 2006). For flowering plants, interactions with insects involve mutualistic associations with pollinators that mediate reproduction, as well as antagonistic interactions with a speciose group of herbivores (Schoonhoven *et al.* 2005, Harder & Barrett 2006). To maximize fitness, flowering plants in nature are challenged to balance investments between reproduction and defence against herbivores. Plants can resist herbivore attack with responses that affect the performance, behaviour and survival of their attackers (Price *et al.* 1980, Schoonhoven *et al.* 2005, Dicke & Baldwin 2010). Reproduction is mediated by the activities of pollinators for two out of three angiosperm plant species (Schoonhoven *et al.* 2005, Raguso 2008, Schiestl *et al.* 2010) and to maximize fitness, flowering plants respond to pollination by insects (Rodriguez-Saona *et al.* 2011, Lucas-Barbosa *et al.* chapter 5). Plant reproductive strategies and defence mechanisms are intrinsically linked and investments in defence and reproduction by plants have long been predicted to trade-off (Herms & Mattson 1992). This trade-off is hypothesised to occur in two main ways: first, when resources allocated to reproduction cannot be allocated to defence and *vice versa*; second, when the attraction of carnivorous insects – so-called indirect plant defence – conflicts with the attraction of pollinators that mediate reproduction. To test these hypotheses, it is necessary 1) to investigate induced plant resistance and allocation of resources by plants, within the same system, and 2) to test if indirect plant resistance can negatively affect the behaviour of pollinators that mediate plant reproduction. Most studies that investigated plant defensive strategies have focused on plants in the vegetative stage (Dicke & Baldwin 2010, Mumm & Dicke 2010); therefore, the link with plant fitness is often missing (van Loon *et al.* 2000, Fritzsche-Hoballah & Turlings 2001, Smallegange *et al.* 2008, Schuman *et al.* 2012), while, of course, positive selection on defensive traits implies a plant fitness benefit.

The aim of this thesis project was to address plant responses induced by herbivory and activities of pollinators while assessing fitness consequences for two of the organisms involved – the plant and the herbivore. My research explored how plants in the flowering stage respond to herbivores, and what the consequences are for mutualistic interactions of plants with carnivorous and pollinating insects. Here, I discuss the ecological significance of plant responses to herbivores and pollinators, placing the findings of this thesis in a wider perspective. I propose a framework within which research questions are articulated to guide the research field to integrate studies of plant defence and reproduction.

Herbivore-induced responses by plants in the flowering stage

In the context of the defence-reproduction trade-off it has been questioned whether plants in the flowering stage could in fact mount defences against herbivores or whether responses were restricted to early developmental stages (Herms & Mattson 1992, Hare 2011). Plant defence is influenced by ontogeny (Barton & Koricheva 2010), and it has recently become clear that plants in the flowering stage do respond to herbivore attack (Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011, Pareja *et al.* 2012, Lucas-Barbosa *et al.* 2013, Bruinsma *et al.* 2014, Schiestl *et al.* 2014, Lucas-Barbosa *et al.* chapter 5). Herbivory can change plant chemistry, influence plant morphology, reproduction, and phenology, and these changes allow for a number of plant-mediated interactions with organisms at different trophic levels (Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011). Insect herbivores can affect plant development by altering speed of reproduction and allocation of resources (Lucas-Barbosa *et al.* 2013, McCormick *et al.* 2013, Lucas-Barbosa *et al.* chapter 7). Herbivory can affect flower traits such as volatile emission and floral rewards (Strauss *et al.* 1996, Lehtila & Strauss 1999, Pareja *et al.* 2012, Lucas-Barbosa *et al.* 2013, Bruinsma *et al.* 2014, Schiestl *et al.* 2014), and influence the behaviour of the plant-associated insect community including that of pollinators (Lucas-Barbosa *et al.* 2013, Schiestl *et al.* 2014), and carnivores (Dannon *et al.* 2010, Pareja *et al.* 2012, Lucas-Barbosa *et al.* 2014, Schiestl *et al.* 2014). Ultimately, responses of flowering plants to herbivory can positively or negatively affect plant fitness (Agrawal 1998, Wise & Cummins 2007, Zangerl & Berenbaum 2009, Cardel & Koptur 2010, Lucas-Barbosa *et al.* 2013).

Defence strategies: there is more to it than plant secondary metabolism

Plant resistance traits can deter, repel or ultimately kill the plant attacker (Schoonhoven *et al.* 2005, Agrawal 2011, Fornoni 2011). Mechanisms that allow plants to compensate for the loss of tissues or damage caused by herbivores have also repeatedly evolved across plants species (Strauss & Agrawal 1999, Schoonhoven *et al.* 2005, Agrawal 2011, Fornoni 2011). Induced plant resistance to herbivory is generally associated with changes at the level of plant secondary metabolism (Schwachtje & Baldwin 2008). However, changes in primary plant metabolites can also confer resistance (Schwachtje & Baldwin 2008, Zhu-Salzman *et al.* 2008), or promote physiological changes that may eventually protect plants against their attackers (Bazot *et al.* 2005, Schwachtje & Baldwin 2008, Lucas-Barbosa *et al.* 2013).

Herbivore attack alters source-sink relationships, and allocation of resources into different tissues can render plants more tolerant to herbivory (Schwachtje & Baldwin 2008, Pashalidou *et al.* 2013, Robert *et al.* 2014). Plants can store resources in roots and invest in re-growth of vegetative tissues when herbivore pressure aboveground is lower, or reallocate resources to aboveground parts when roots are attacked (Briske *et al.* 1996, Derner & Briske 2001, Bazot *et al.* 2005, Schwachtje & Baldwin 2008, Bazot *et al.* 2013, Robert *et al.* 2014). Upon herbivory, plants can also invest in reproductive tissues to produce offspring before flowers are consumed. *Brassica nigra* plants respond to egg deposition by *P. brassicae* butterflies by speeding up flower and seed production (Lucas-Barbosa *et al.* 2013, Pashalidou *et al.* 2013); this benefits the plants because after hatching, caterpillars quickly move to the inflorescence where they consume flowers, but not seeds (Lucas-Barbosa *et al.* 2013).

We cannot fully understand how plant fitness is maximised when we focus only on resistance traits. It is important to investigate how herbivory influences allocation of resources by plants and to what extent plants can tolerate herbivory. In theory, it is reasonable to think that a resistant plant does not experience selection to tolerate herbivory, and that a tolerant plant does not experience selection to resist herbivory (Agrawal 2011). However, out there in nature, there are neither 100% resistant plants nor 100% tolerant plants. Thus, to fully understand how plants maximize fitness it is important to investigate herbivore-induced plant resistance and allocation of resources by plants upon herbivory, within the same study system (Fig. 1).

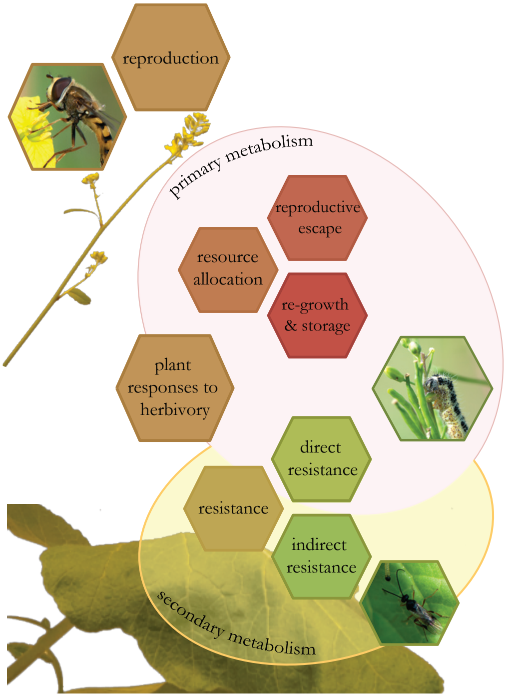

Fig. 1 Herbivore-induced plant resistance and allocation of resources by plants upon herbivory should be investigated within the same study system.

Allocation of resources in response to herbivore attack can render plants more tolerant, and are related to changes in primary plant metabolism. Resistance traits encompass both primary and secondary plant metabolism.

Can carnivore attraction conflict with pollinator attraction: when and how?

Plant responses to herbivore attack can enhance the effectiveness of carnivorous insects. There is indeed ample evidence that such plant responses are exploited by natural enemies of the herbivores, particularly when regarding the production of herbivore-induced plant volatiles (HIPV) (Mumm & Dicke 2010). The fitness benefits associated with the production of HIPVs are less evident and led researchers in this field to question 1) whether this indirect form of plant resistance is, in fact, a defensive strategy of plants under attack (Dicke & Baldwin 2010, Hare 2011), and 2) if so, how do HIPVs influence mutualistic interactions of plants with pollinators (Kessler & Halitschke 2009, Dicke & Baldwin 2010, Lucas-Barbosa *et al.* 2011). In fact, herbivory can induce changes in plants in the flowering stage, and these do not relate only to cues generally exploited by flower visitors (Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011, Bruinsma *et al.* 2014), but also involve changes in the rewards that flowers provide to pollinating insects (Kessler & Baldwin 2007, Bruinsma *et al.* 2014). Changes in flower traits in response to herbivory can alter pollinator visitation frequency, which in turn may affect pollination success, and seed set. First studies have investigated effects of herbivore-induced responses on pollinator behaviour, and, not surprising, results are variable (Kessler & Halitschke 2009). Herbivory may have positive, negative or no influence on pollinator behaviour (Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011). When herbivory increases the attraction of pollinators, we can expect that a threatened plant can benefit from enhanced interactions with mutualistic insects (Strauss & Murch 2004). However, most studies so far have recorded a negative effect of herbivory on pollinator behaviour (Kessler & Halitschke 2009). A potential conflict between induced plant responses and plant reproduction may be associated with modified pollinator behaviour if negative effects of herbivore-induced responses on pollinator behaviour lead to negative consequences for plant fitness (Kessler & Halitschke 2009, Lucas-Barbosa *et al.* 2011). However, the evidence collected so far has rarely been connected to plant fitness (Kessler & Halitschke 2009). Here, I emphasise the need to connect the study of induced responses to herbivory with that of plant-pollinator associations, and ultimately to plant fitness.

It is crucial to investigate as well what the basis is for the variation in herbivore-induced responses by plants and their effects on pollinator behaviour. The diversity of plant defensive strategies against herbivores, and specificities in feeding preferences of herbivores and pollinators offers a wide range of almost unexplored subjects for research, from both the plant and insect perspectives. For instance, herbivores belonging to different feeding guilds or that preferentially feed on distinct parts of plants can induce different plant responses (Stam *et al.* 2014), and influence pollinators in different ways. Studies focusing on this subject should investigate a range of herbivorous insects that differ in the feeding mode or preference, and it is crucial to focus on those that in nature share the same flowering host plant. Moreover, it is also important to consider that our expectations and hypotheses will likely differ when focusing on generalized or on specialized plant-pollinator associations. Although in specialized plant-pollinator associations, pollinator responses can be more directly connected to plant fitness, perhaps it is from generalized systems that we can learn the most. It would not be surprising, but still fascinating, to discover that diversity in flower chemistry and flower-associated organisms, including pollinators and florivores, are reciprocally linked (Frame 2003, Schiestl & Johnson 2013). I expect that in generalized systems a diverse group of pollinators selects for diversity in flower chemistry and plasticity of responses to herbivores and pollination (Kessler & Halitschke 2007, Lucas-Barbosa *et al.* 2011, Schiestl & Johnson 2013, Dormont *et al.* 2014). Plant species also vary in how they invest in defensive and reproductive strategies (Schwachtje & Baldwin 2008, Agrawal 2011, Fornoni 2011, Lucas-Barbosa *et al.* 2013, Pashalidou *et al.* 2013, Robert *et al.* 2014). Studies addressing a range of plant species that significantly vary in defensive or reproductive strategies can help us to understand when plant defensive traits conflict with pollinator attraction. Results of this thesis show that herbivory can influence flower traits and rewards, and these changes can influence pollinator preferences (Bruinsma *et al.* 2014). In the field, overall interactions with pollinators were maintained and herbivore-infested plants produced as many seeds as control *B. nigra* plants (Lucas-Barbosa *et al.* 2013). Upon herbivory, *B. nigra* plants reallocate resources to flowers and increase the production of secondary metabolites particularly in flower tissues (Bruinsma *et al.* 2014, Lucas-Barbosa *et al.* chapter 7), while maintaining interactions with various day-active pollinating insects (Lucas-Barbosa *et al.* 2013). Although some plants enhance investments in reproduction upon herbivory, there are also examples of plant species that, upon herbivore attack, will invest in growth of vegetative tissues or storage of resources in roots (Briske *et al.* 1996, Derner & Briske 2001, Bazot *et al.* 2005, Schwachtje & Baldwin 2008, Bazot *et al.* 2013, Robert *et al.* 2014). I hypothesise that the latter cases lead to negative impacts on interactions with pollinators.

Addressing the underlying mechanisms of plant responses to herbivory and pollination helps to understand how the attraction of carnivores can conflict – or not – with the attraction of pollinators. Flower-associated organisms exploit flower traits, such as odours and flower pigments (Raguso 2008, Schiestl & Johnson 2013) to assess reward quality, and thus, their behaviour and performance can be affected by changes in these flower traits (Lucas-Barbosa *et al.* 2011, Schiestl *et al.* 2014). A smaller flower offers less odour and weaker visual cues than a big flower, and the production of flower odours and pigments can be regulated by common precursors, meaning that changes in pigments might lead to changes in odours and *vice versa* (Dormont *et al.* 2014). Moreover, secondary metabolites that have defensive and reproductive functions are also regulated by common phytohormones and produced by shared biosynthetic pathways (Koornneef & Pieterse 2008, Gershenzon *et al.* 2012, Dicke & van Loon 2014), and the systemic nature of herbivore-induced responses can influence flower traits (Lucas-Barbosa *et al.* 2011). Pollinators too can induce changes in flowers, but these responses are generally local and not systemic (Rodriguez-Saona *et al.* 2011, Lucas-Barbosa *et al.* chapter 5). Changes in flowers upon pollination can render unpollinated flowers of the same plant more attractive to pollinators. We observed that herbivore-induced responses can interfere with responses to pollination that optimise reproduction. Nectar production, for instance, usually ceases after a flower has been pollinated (Rodriguez-Saona *et al.* 2011). As a result, nectar feeders may avoid flowers that provide little of this reward. In *B. nigra*, the odour profile of pollinated plants differs from that of unpollinated plants (Lucas-Barbosa *et al.* chapter 5). However, when plants were exposed to both pollination and herbivory their odour blend could not be distinguished from either the blends of pollinated or unpollinated plants (Lucas-Barbosa *et al.* chapter 5). This reveals a possible mechanism through which herbivore-induced responses can conflict with pollinator attraction. However, this may not necessarily be translated to consequences for plant fitness if flowers are pollinated by various insect species. Pollinators that exploit different rewards also exploit different flower traits (Lucas-Barbosa *et al.* chapter 5), and insects may even exploit different traits when searching for the same reward. Thus, when looking from the plant perspective, fitness consequences of responses to herbivory and pollination need to be investigated in the field, where the pollinator community associated with the plant can be included.

Carnivore attraction as plant defence strategy

Carnivores can exploit plant odours produced in response to herbivore attack to locate prey (Dicke & Baldwin 2010, Mumm & Dicke 2010). The role that carnivores play as a component of plant defence against herbivorous insects has been debated because we lack data showing that there is a plant fitness benefit associated with the production of HIPVs (Dicke & Baldwin 2010, Hare 2011). Indeed, if carnivore attraction, *via* the production of HIPVs, is a plant defence strategy we should expect that selection can be imposed on plants to maintain attraction of carnivores. Along this line, we should also expect that there is selection on the herbivore to avoid carnivores. In fact, there are many examples in the literature suggesting that this is the case, and that herbivores avoid enemy-dense host plants, or even parts of plants where they are more vulnerable to carnivore attack (Ohsaki & Sato 1994, Matter *et al.* 1999, Dicke & Grostal 2001, Gross *et al.* 2004, Mulatu *et al.* 2004, Murphy 2004, Singer *et al.* 2004, Lucas-Barbosa *et al.* 2014). The fitness benefit associated with HIPV production, however, cannot be easily demonstrated (Dicke & Baldwin 2010, Hare 2011). Yet, a few studies provide evidence that a plant fitness benefit can be associated with carnivore attraction (van Loon *et al.* 2000, Fritzsche-Hoballah *et al.* 2002, Smallegange *et al.* 2008, Schuman *et al.* 2012). In *B. nigra,* carnivores are important in maintaining a low herbivore pressure during flowering. In three consecutive years of field experiments, we observed that survival of *P. brassicae* on mustard plants was lower than 5% (Lucas-Barbosa *et al.* 2013, Lucas-Barbosa *et al.* 2014, Lucas-Barbosa *et al.* chapter 7). Not only specialist parasitoids attack caterpillars, but also generalist predators kill a large proportion of larvae, preventing this voracious feeder from compromising plant fitness (Lucas-Barbosa *et al.* 2013, Lucas-Barbosa *et al.* 2014). In fact, in the absence of carnivores, plants suffered from herbivory (Lucas-Barbosa *et al.* chapter 7). The data collected in this manipulative field experiment do not prove that plants benefit from producing HIPVs, but they do support the importance of carnivores as a component of a plant's defence strategy (Dicke & Baldwin 2010, Hare 2011).

Concluding notes and a way forward

My research contributes to our understanding of how plants in the flowering stage respond to herbivore attack and what the consequences are for the fitness of the plant and survival of the herbivore. Upon attack by specialist herbivores, *B. nigra* plants maintain interactions with pollinators and carnivores, sustaining reproductive output. This work revealed some of the underlying mechanisms that allow this short-lived brassicaceous plant to balance investments in defence and reproduction, including mechanisms of plant resistance and re-allocation of resources by plants.

Many studies have been able to identify mechanisms of plant resistance or re-allocation of resources by plants, but these two strategies by which plants may maximise fitness have been rarely investigated within the same study system. Particularly, when regarding plant resistance against insects, we have observed that complexity in this area of research had increased vertically, and many studies have identified mechanisms through which insects up to the 4th trophic level are affected by plant resistance traits (Ode 2006, Harvey *et al.* 2007). However, these studies did not or could not demonstrate whether these mechanisms indeed result in a fitness benefit for the plant. This is much related to the fact that almost all of this research has been done with plants in the vegetative stage, and has rarely been taken all the way until plant fitness could be quantified. The ultimate challenge is to assess plant fitness benefit of the expression of defensive traits that can be quantified above the natural background noise observed in field conditions. To increase our understanding of how multiple defence strategies evolved in plants, we should perhaps consider increasing complexity horizontally, and use natural variation among plant species within a family to assess the ecological costs associated with plant responses to herbivores and pollinators through a comparative approach (Weber & Agrawal 2012).

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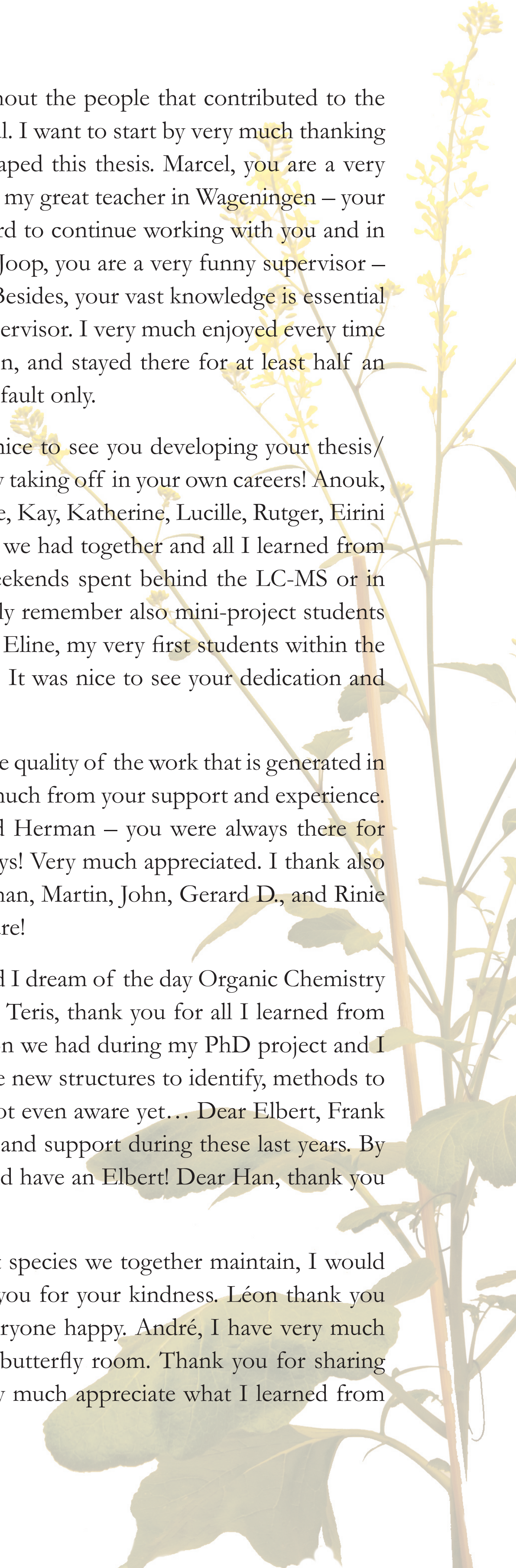

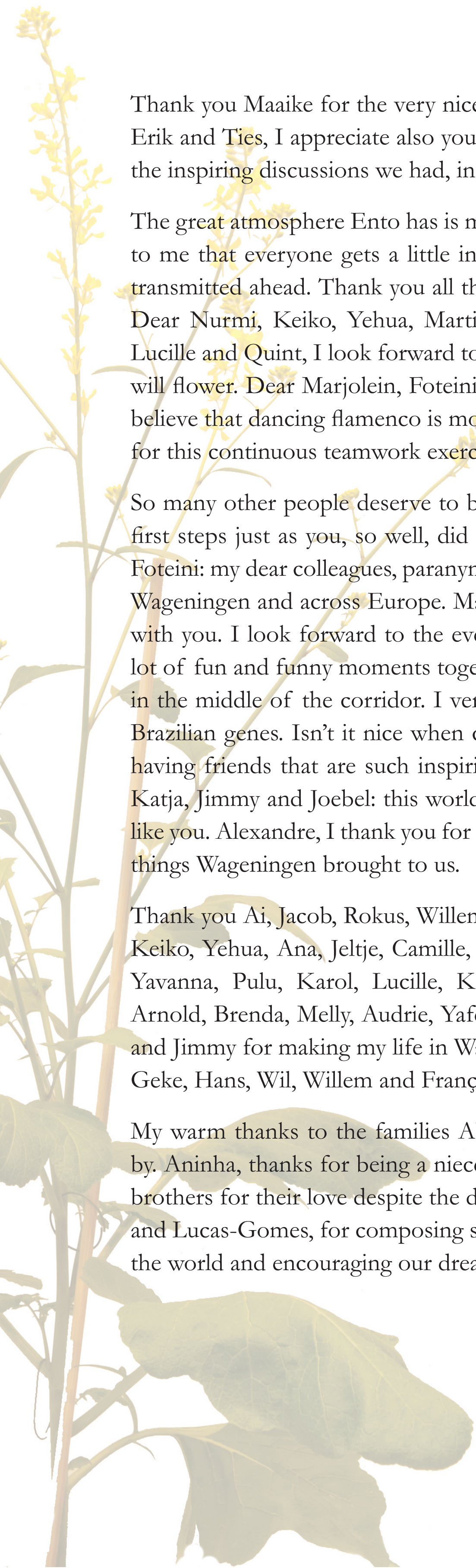

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Summary

Plants are under natural selection to maximize fitness, and most flowering plants rely on insect pollinators for reproduction. However, to be able to reproduce plants need to survive the battle against insect herbivores. Plants must, therefore, balance the investments in defences against herbivores with investments in growth/reproduction, and these aspects have been expected to trade-off. A trade-off between plant defence and reproduction is expected when: 1) resources that are allocated to reproduction cannot be allocated to defence and *vice versa*; 2) the attraction of carnivorous insects − so-called indirect defence − conflicts with attraction of pollinators that mediate reproduction. However, despite this potential trade-off, plant reproduction and plant defence have typically been investigated in isolation. In fact, induced plant defences cannot be fully understood when disconnected from plant-pollinator interactions because selection on defensive traits against herbivores implies a plant fitness benefit.

This thesis explores the fitness consequences of plant responses induced by herbivores in the context of plant-mediated interactions with carnivorous and pollinating insects. The study used the Black mustard *Brassica nigra* and the Large Cabbage White butterfly *Pieris brassicae* as the main model organisms. *Brassica nigra* is a wild annual brassicaceous species, and grows as a successional plant standing in high densities. This wild species has high concentrations of defensive compounds such as phenolics and glucosinolates that protect plants from generalist herbivores. This plant species is, therefore, mainly attacked by specialist herbivores such as *P. brassicae*. *Pieris brassicae* is a gregarious species, and caterpillars will initially feed on leaves where eggs were deposited by the mother butterfly, but the second instar larvae move to the flowers. Caterpillars of *P. brassicae* are voracious feeders and consume entire flowers in large numbers.

At the start of this project, the literature on herbivore-induced responses was reviewed with focus on the consequences for insect-flower interactions, *i.e.* in the context of the trade-off between defence and reproduction. More specifically, it was reviewed how herbivore-induced volatiles emitted by plants in the flowering stage can influence the plant-associated insect community, including the effects of herbivory on host-plant selection by adult herbivores, host-location behaviour of parasitoids, and foraging preferences of pollinators. In this literature review, it was identified that the role of flower volatiles in the interactions between plants and insect pollinators has received increased attention over the last decade. However, studies addressing both herbivore-induced plant volatiles and pollinator behaviour were rare, despite the fact that in a number of plant species herbivory is known to affect flower traits, including size, nectar secretion and composition. I concluded then that approaches to integrate the study of plant defences and pollination were, and are still to date, essential to advance plant biology, in particular in the context of the trade-off between defence and growth/reproduction.

A common garden experiment was then designed as a kick-off for this thesis project. The main goal of this field experiment was to evaluate the overall effects of herbivory by *P.*

brassicae on plant fitness. Responses of *B. nigra* plants to herbivore exposure were investigated since egg deposition, throughout larval development. The herbivores that were deliberately introduced were exposed to naturally occurring predators and parasitoids, and dispersal and mortality of caterpillars during the experiments were recorded. Furthermore, the effects of infestation by the specialist *P. brassicae* on: 1. pollinator behaviour; 2. volatile emission; 3. timing and number of seeds produced were quantified. Remarkably, it was observed that before eggs had hatched, infested plants speeded up seed production. The caterpillars feed on flowers but not on seeds. Thus, by accelerating seed production, plants prevented consumption of flowers, and effectively defended themselves against the herbivores. Overall, interactions with pollinators were maintained, and infested plants produced as many or more seeds than non-infested control plants. Herbivory can, however, influences pollinator visitation differently at the species level, and also depending on whether leaves or flowers were attacked by the herbivores. Thus, in chapters 4 and 5 it was investigated whether and how induced plant responses to herbivory can lead to changes in pollinator behaviour.

In Chapter 4, the systemic effects of herbivore damage to leaves on floral reward and flower traits exploited by pollinators were addressed, considering the role of defensive chemistry against herbivores on pollinator behaviour. Pollinator visitation is influenced by the quality and quantity of pollinator rewards, such as floral nectar, and by flower traits that can be associated with the value of such reward. Therefore, the aim in this chapter was to infer whether changes in odours exploited as cues by pollinators, could be associated with actual changes in quantity and quality of nectar offered by *B. nigra* flowers. Herbivory influenced both flower volatile emission and sugar content in nectar. Interestingly, volatile emission by flowers changed upon feeding by herbivores on the leaves, whereas, , volatile emission by leaves did not significantly differ between infested and non-infested flowering plants. The frequency of flower visits by pollinators was generally not influenced by herbivory, but honeybees and butterflies spent less time visiting flowers of herbivore-infested plants that provided sweeter nectar.

In chapter 5 the mechanisms through which herbivore-induced responses by plants can affect pollinator behaviour were further examined. This chapter addresses not only induced phytochemical responses to herbivory, but also plant responses to pollination. Plants can respond to the activities of pollinators and herbivores, and the same classes of plant secondary metabolites associated with induced responses to herbivory can also be associated with pollinator attraction. In this chapter, it was investigated how responses of *B. nigra* plants to pollination and insect herbivory affect the behaviour of flower visitors when caterpillars were feeding on the leaves, or on the flowers of *B. nigra* plants. Moreover, this chapter addresses how these plants respond to pollination and insect herbivory in terms of the production of volatile and non-volatile compounds, and whether plant responses to herbivory interfere with responses to pollination. Results show that butterflies used different cues when searching for an oviposition site or a nectar source. Syrphid flies visited preferably recently opened flowers, and previous pollination did not influence their behaviour. Plants responded to pollination and herbivory with changes in the profile of volatiles and non-volatiles of *B. nigra* flowers; this can potentially influence the colour-odour association exploited by pollinators. Furthermore, the systemic nature of herbivore-induced plant responses can interfere with local responses to pollination that can be important in optimizing plant reproductive success. The results of chapters 4 and 5 are discussed in the context of the trade-off between plant defence and pollinator attraction, and I conclude that both herbivores and pollinators induced important phenotypic changes in flowers. Moreover, different pollinator species can be differently affected by herbivore-induced responses depending, for instance, on the reward they are searching for. Therefore, responses to pollination and herbivory must be addressed in an integrated manner because, in nature, plants are exposed to herbivores and pollinators at the same time, and at times, by various pollinator species.

In Chapter 6, the herbivore perspective was taken to investigate why *P. brassicae* caterpillars feed on the flowers. The survival of insect herbivores is typically constrained by food choice and predation risk. In this context, this chapter addresses whether flowers provided caterpillars with a refuge from their natural enemies, and what the consequences were for the survival of *P. brassicae* when feeding on flowering plants. Field and greenhouse experiments were used to investigate how flowers influence host-plant selection by adult herbivores, and subsequent interactions of the herbivore with parasitoids and predators on different parts of the flowering plant. The results show that by moving to flowers, caterpillars escaped from the parasitoid. Flowers are nutritionally superior when compared with leaves, and caterpillars develop faster when feeding on flowers. However, late-stage caterpillars were intensively preyed upon by social wasps, irrespective of whether they fed on leaves or flowers. The results are discussed in the context of theories of diet breadth and enemy-free space, and in terms of the selective pressures and constraints on herbivore survival in nature, and I conclude that flower preference by *P. brassicae* is more likely driven by nutritional advantages and reduced risk of parasitism when feeding on flowers, than by the risk of being killed by generalist predators. Overall, in *B. nigra,* carnivores are important in maintaining a low herbivore pressure during the flowering stage. In two consecutive years of field experiments, I had observed that carnivorous insects can kill up to 95% of *P. brassicae* caterpillars on flowering *B. nigra*. Thus, chapter 7 addresses the role of carnivorous insects as a defence strategy in *B. nigra,* and how these plants allocate resources after herbivore attack. The overall aim was to investigate how *B. nigra* plants balanced investments between reproduction and defensive strategies against the specialist herbivore *P. brassicae*. The data show that plants responded to caterpillar feeding by allocating resources to reproduction, and compensated for fitness loss due to herbivory in terms of seed production when interactions with carnivores were maintained. However, in the absence of carnivores, *B. nigra* did not compensate for the damage caused by *P. brassicae* caterpillars. These data support the importance of carnivores as a component of a plant's defence strategy.

The data presented in this thesis contribute to our understanding of how plants in the flowering stage respond to herbivore attack and what the consequences are for plant fitness and herbivore survival. Upon attack by specialist herbivores, *B. nigra* plants maintain interactions with pollinators and insect carnivores, and overall plant fitness is sustained. This work revealed some of the underlying mechanisms that allow this short-lived brassicaceous plant to balance investments in defence and reproduction, including mechanisms of plant resistance and re-allocation of resources by plants. I emphasise that to fully understand how plant defence strategies evolved it is necessary to integrate studies on plant-herbivore and plant-pollinator interactions. Ultimately, such studies need to be taken all the way to the level of plant fitness. The findings of this thesis, together with recently published studies by others, provide the first steps in this direction.

Samenvatting

Planten staan onder natuurlijke selectie om hun reproductie te maximaliseren en de meeste bloeiende planten zijn afhankelijk van bestuivers voor hun voortplanting. Om te kunnen reproduceren moeten planten echter de aanval van herbivore insecten overleven. Planten moeten daarom een balans vinden in hun investeringen in verdediging tegen planteneters en hun investeringen in groei/reproductie. De verwachting is dat deze investeringen elkaar wederzijds uitsluiten. Een conflict tussen de verdediging van planten en hun voortplanting wordt verwacht wanneer: 1) de middelen die worden uitgetrokken voor voortplanting niet kunnen worden geïnvesteerd in verdediging en *vice versa*; 2) de aantrekking van natuurlijke vijanden van planteneters — zogenaamde indirecte verdediging — interfereert met de aantrekking van bestuivers die nodig zijn voor de voortplanting. Ondanks dit potentiële conflict, worden voortplanting en verdediging van planten grotendeels apart bestudeerd. Toch kunnen geïnduceerde verdedigingsmechanismen van planten niet volledig worden begrepen wanneer zij worden losgekoppeld van plant-bestuiver interacties omdat natuurlijke selectie op verdedigingskenmerken impliceert dat de verdediging een fitness voordeel heeft.

Dit proefschrift rapporteert over studies naar de gevolgen van de reacties van planten op vraatschade voor de interacties van planten met de vijanden van de planteneters en bestuivende insecten. Zwarte mosterd, *Brassica nigra,* en het Groot Koolwitje, *Pieris brassicae,* staan centraal in de studie. *Brassica nigra* is een wilde eenjarige kruisbloemige en groeit als een vroege successie-plant. De planten komen in hoge dichtheden voor. Deze wilde plant heeft hoge concentraties van verdedigingsstoffen zoals fenolen en glucosinolaten, die de plant beschermen tegen generalistische herbivoren. Deze plantensoort wordt daarom vooral aangevallen door gespecialiseerde herbivoren zoals *P. brassicae*. *Pieris brassicae* vlinders leggen hun eieren in groepen en de rupsen voeden zich in eerste instantie met de bladeren waarop de eieren werden afgezet door hun moeder; na hun vervelling tot het tweede larvestadium verhuizen de rupsen naar de bloemen. Rupsen van *P. brassicae* zijn vraatzuchtig en consumeren grote aantallen bloemen.

Aan het begin van dit project werd een overzicht gemaakt van de literatuur over de reacties van planten op insectenvraat, met nadruk op de gevolgen voor interacties tussen bloemen en insecten, in het kader van het conflict tussen verdediging en voortplanting. Meer in het bijzonder werd onderzocht hoe de geurstoffen die bloeiende planten maken in reactie op vraatschade de insecten beïnvloeden die met de plant geassocieerd zijn, inclusief de effecten van vraatschade op waardplantselectie door volwassen herbivoren, gastheerlocalisatie door carnivore insecten en foerageerkeuzes van bestuivers. Deze literatuurstudie liet zien dat de rol van bloemgeuren in de interacties tussen planten en bestuivende insecten meer en meer aandacht heeft gekregen in de afgelopen tien jaar. Studies naar de geuren die planten maken in reactie op insectenvraat en ook de reacties van bestuivers onderzochten zijn echter zeldzaam. Toch is er wel enige kennis van effecten van insectenvraat op bloemkenmerken, inclusief bloemgrootte, nectarproductie en nectarsamenstelling. Op basis van deze literatuurstudie concludeerde ik dat een geïntegreerde studie van plantenverdediging en bestuiving belangrijk is om de kennis van de biologie van planten te bevorderen, in het bijzonder in het kader van het conflict tussen verdediging en groei/voortplanting.

Een veldexperiment werd vervolgens opgezet als start van dit project. Het belangrijkste doel van dit veldexperiment was te onderzoeken wat de effecten zijn van vraat door rupsen van het Groot Koolwitje op de reproductie van de planten. Reacties van *B. nigra* planten op blootstelling aan het Groot Koolwitje werden onderzocht vanaf ei-afzetting en gedurende de hele larvale ontwikkeling. De geïntroduceerde herbivoren werden blootgesteld aan hun natuurlijke vijanden (predatoren en sluipwespen) en de verspreiding en mortaliteit van rupsen tijdens de experimenten werden geregistreerd. Bovendien werd het effect van blootstelling aan de specialist *P. brassicae* onderzocht met betrekking tot: 1. gedrag van bestuivende insecten; 2. de productie van geurstoffen door de planten; 3. moment van zaadproductie en het aantal geproduceerde zaden. Opmerkelijk was de waarneming dat nog vóór de eieren uitgekomen waren, de planten een versnelde zaadproductie lieten zien. De rupsen eten de bloemen maar niet de zaden. Door het versnellen van de zaadproductie, konden planten zichzelf effectief verdedigen tegen de herbivoren. De blootstelling aan herbivoren leidde niet tot grote effecten op de interacties met bestuivers en planten die aan herbivoren waren blootgesteld produceerden net zo veel of meer zaden dan controleplanten. De effecten van herbivorenvraat op bestuivende insecten kan echter verschillen tussen soorten bestuivers en is ook afhankelijk van of de bladeren of bloemen werden aangevallen door de herbivoren. Zo is in hoofdstukken 4 en 5 onderzocht of en hoe de reacties van planten op herbivorie kan leiden tot veranderingen in het gedrag van bestuivers.

In hoofdstuk 4 is onderzocht wat de systemische effecten zijn van vraat aan bladeren op kenmerken van bloemen, die van belang zijn voor bestuivers. Bezoek door bestuivers wordt beïnvloed door de kwaliteit en kwantiteit van beloningen die bloemen aan bestuivers bieden, zoals nectar, en bloemkenmerken die geassocieerd zijn met dergelijke beloningen. Daarom was het doel van dit hoofdstuk om te bepalen of veranderingen in geuren die door bestuivers gebruikt worden, geassocieerd zijn met de veranderingen in de hoeveelheid en de kwaliteit van de nectar van de bloemen. Herbivorie beïnvloedt zowel de emissie van bloemgeuren als het suikergehalte in de nectar. Interessant is dat de geuremissie door bloemen veranderde na herbivorenvraat op de bladeren, terwijl opmerkelijk genoeg de geuremissie door bladeren niet verschilde tussen bloeiende planten met en zonder vraat aan de bladeren. De frequentie van de bloembezoeken door bestuivers werd in het algemeen niet beïnvloed door herbivorie, maar bijen en vlinders besteedden minder tijd aan het bezoeken van bloemen op planten met herbivoren, die zoetere nectar bevatten.

Hoofdstuk 5 laat zien hoe de reacties van planten op herbivorie de interacties van planten met bestuivers beïnvloeden. In dit hoofdstuk worden niet alleen fytochemische reacties op herbivorie onderzocht, maar ook reacties van planten op bestuiving. Planten kunnen reageren op de activiteiten van bestuivers en herbivoren, en dezelfde klassen van secundaire plantenstoffen die een rol spelen in de inductie door herbivorie kunnen ook geassocieerd zijn met de aantrekking van bestuivers. In dit hoofdstuk werd onderzocht hoe de reacties van *B. nigra* planten op bestuiving en insectenvraat het gedrag beïnvloeden van bloembezoekers wanneer rupsen aten van de bladeren of de bloemen van deze planten. Bovendien komt aan de orde hoe bestuiving en insectenvraat de productie van plantengeuren en niet-vluchtige plantenstoffen beïnvloeden. Ook werd onderzocht of de reactie van planten op herbivorie interfereert met de reactie op bestuiving. De resultaten laten zien dat vlinders verschillende signalen gebruiken wanneer ze zoeken naar een plaats om eieren te leggen of naar een nectarbron. Zweefvliegen hebben een voorkeur voor recent geopende bloemen, en eerdere bestuiving beïnvloedt hun gedrag niet. Planten reageren op bestuiving of insectenvraat met veranderingen in het mengsel van vluchtige en niet-vluchtige stoffen dat *B. nigra* bloemen produceren. Dit kan mogelijk invloed hebben op de associatie van kleuren en geuren door bestuivers. Bovendien kan het systemische karakter van herbivoor-geïnduceerde plantenreacties interfereren met lokale reacties van de plant op bestuiving die belangrijk kunnen zijn voor het optimaliseren van het reproductief succes van de planten. De resultaten van de hoofdstukken 4 en 5 worden besproken in het kader van het conflict tussen de verdediging van planten en de aantrekking van bestuivers. Ik concludeer dat zowel herbivoren als bestuivers belangrijke fenotypische veranderingen in bloemen teweeg kunnen brengen. Bovendien kunnen verschillende soorten bestuivers elk op eigen wijze worden beïnvloed door de reacties van planten op herbivorie. Dit is mogelijk afhankelijk van bijvoorbeeld het type beloning dat ze zoeken. Het is daarom belangrijk om de reacties van planten op bestuiving en herbivorie op een geïntegreerde manier te onderzoeken. Immers, in de natuur zijn planten tegelijkertijd blootgesteld aan herbivoren en bestuivers en soms ook aan verschillende soorten bestuivers.

In hoofdstuk 6 wordt vanuit het perspectief van herbivoren onderzocht waarom *P. brassicae* rupsen zich voeden met de bloemen van zwarte mosterdplanten. De overleving van herbivore insecten wordt meestal beïnvloed door voedselkeuze en predatierisico. In deze context richt dit hoofdstuk zich op de vraag of bloemen rupsen een vrijplaats bieden ten opzichte van hun natuurlijke vijanden, en wat de gevolgen zijn voor de overleving van *P. brassicae* wanneer deze eten op bloeiende planten. Veld- en kasexperimenten werden uitgevoerd om na te gaan hoe bloemen de waardplantselectie door vlinders beïnvloeden, alsmede de daaropvolgende interacties van de rupsen met sluipwespen en roofvijanden op verschillende delen van de bloeiende plant. De resultaten laten zien dat door zich te verplaatsen naar de bloemen, jonge rupsen ontsnappen aan sluipwespen. Bloemen hebben een hogere voedingswaarde dan bladeren en rupsen ontwikkelen zich sneller wanneer ze zich voeden met bloemen dan met bladeren. De rupsen werden echter intensief weggeroofd door sociale wespen, ongeacht of de rupsen aten van bladeren of bloemen. De resultaten worden besproken in de context van theorieën over voedselrange en het ontlopen van vijanden, en met betrekking tot natuurlijke selectiedruk en de druk op de overleving van herbivoren in de natuur. Ik concludeer dat bloemvoorkeur van *P. brassicae* waarschijnlijk meer beïnvloed is door nutritionele voordelen en een verminderd risico van parasitisme tijdens het eten van bloemen, dan door het risico om te worden gedood door generalistische roofvijanden. Over het algemeen zijn carnivoren voor zwarte mosterdplanten belangrijk om de druk van herbivorie laag te houden tijdens de bloeifase. In twee opeenvolgende jaren van veldexperimenten, registreerde ik dat de mortaliteit van *P. brassicae* rupsen door carnivore insecten op bloeiende *B. nigra* kan oplopen tot wel 95%. Hoofdstuk 7 richt zich daarom op de rol van carnivore insecten in de verdedigingsstrategie van *B. nigra*, en hoe deze planten hun reserves verdelen na insectenvraat. Het algemene doel was om te onderzoeken hoe *B. nigra* planten hun investeringen verdelen over voortplanting en verdediging tegen de specialistische herbivoor *P. brassicae*. De onderzoeksresultaten tonen aan dat planten reageren op rupsenvraat met een investering in voortplanting, en daarmee compenseren voor fitnessverlies als gevolg van herbivorie in aanwezigheid van de vijanden van de rupsen. In de afwezigheid van de vijanden van de rupsen, compenseren *B. nigra* planten echter niet voor de schade veroorzaakt door de rupsen. Deze resultaten laten zien dat carnivoren een belangrijk onderdeel zijn van de verdedigingsstrategie van *B. nigra* planten.

De onderzoeksresultaten die in dit proefschrift gepresenteerd worden dragen bij aan ons begrip van hoe planten in de bloeifase reageren op de aanval door insecten, en wat de gevolgen zijn voor zaadproductie van de planten en de overleving van de plantenetende insecten. Na de aanval door gespecialiseerde plantenetende insecten, worden de interacties van de plant met bestuivers en carnivore insecten in stand gehouden, en blijft de gerealiseerde fitness op het niveau van onaangetaste planten. Dit onderzoek heeft een aantal van de onderliggende mechanismen opgehelderd die het mogelijk maken dat deze eenjarige kruisbloemige plant de balans bewaart tussen investeringen in verdediging en reproductie, met inbegrip van mechanismen van resistentie in planten en re-allocatie van middelen door planten. Ik benadruk dat voor een goed begrip van de evolutie van plantenverdediging het noodzakelijk is om de interacties van planten met zowel herbivoren als met bestuivers op een geïntegreerde manier te onderzoeken. Uiteindelijk moeten deze studies ook de effecten op plantfitness omvatten. De bevindingen van dit proefschrift, samen met recente studies van anderen, vormen de eerste stappen in deze richting.

Resumo

As plantas estão em constante processo de seleção natural para maximizar fitness. A maioria das angiospermas dependem de insetos polinizadores para a reprodução. Entretanto, para se reproduzir ou alcançar a fase reprodutiva, as plantas necessitam, primeiramente, sobreviver ao ataque de insetos herbívoros. Assim, para as plantas é fundamental manter o equilíbrio entre os investimentos em defesas contra herbívoros e investimentos crescimento/reprodução, e esses aspectos são esperados a se contraporem. Um conflito entre defesa e reprodução quando: 1) recursos primários que são alocados para a reprodução não podem ser alocados à defesa e *vice-versa*; 2) a atração de insetos carnívoros – que compõem a defesa indireta da planta − conflitam com a atração de polinizadores que auxiliam a reprodução. No entanto, apesar desse possível conflito entre reprodução e defesa, os mesmos têm sido tipicamente investigados de forma isolada. Na verdade, mecanismos de defesa induzidas nas plantas não podem ser inteiramente compreendidos quando estudados separadamente das interações entre plantas e agentes polinizadores, porque a seleção de características defensivas contra herbívoros implica diretamente em benefício para a planta em termos reprodutivos.

A presente tese de doutorado explora as consequências da capacidade das plantas em responder ao ataque de insetos herbívoros no contexto das interações mediadas pelas plantas com insetos carnívoros e polinizadores. Como principais organismos modelo foram utilizados a mostarda-preta, *Brassica nigra* (L.), e a borboleta da couve *Pieris brassicae* (L.), uma espécie especializada em plantas da família Brassicaceae. A mostarda-preta *B. nigra* é uma espécie anual, selvagem e que cresce como planta pioneira em densidades elevadas. Esta espécie produz altas concentrações de compostos de defesa, tais como fenóis e glucosinolatos, os quais protegem a planta contra herbívoros generalistas. Essa planta é, portanto, atacada principalmente por herbívoros especializados, como *P. brassicae*. O lepidóptero *P. brassicae* tem uma fase inicial gregária, sendo que as lagartas inicialmente se alimentam de folhas, substrato usado para a colocação dos ovos, e no segundo instar larval passam a se alimentar das flores. As lagartas de *P. brassicae* são vorazes, consumindo as flores completamente e em grande quantidade.

No início deste projeto, a literatura sobre as respostas induzidas por herbívoros foi revista com foco nas consequências para as interações inseto-flor, ou seja, no contexto de uma possível conflito entre defesa e reprodução. Mais especificamente, esta revisão da literatura avaliou como compostos orgânicos voláteis produzidos pela planta durante o ciclo reprodutivo, e em resposta a ataque dos herbívoros, podem influenciar a comunidade de insetos associada à planta. Aspectos como efeitos da herbivoria na seleção da planta hospedeira pelos herbívoros adultos, na localização das plantas pelos predadores e parasitoides, e nas preferências dos polinizadores foram discutidos. Nesta revisão de literatura, identificou-se que o papel dos compostos orgânicos voláteis produzidos pela flores nas interações entre plantas e insetos polinizadores, receberam mais atenção nesta última década. Entretanto, estudos que ao mesmo tempo descrevem a produção de voláteis em plantas induzida por herbívoros, e o consequente efeito no comportamento dos polinizadores foram raramente relatados. Várias espécies de insetos, no entanto, sabidamente afetam aspectos morfológicos e a composição

química das flores incluindo, por exemplo, forma, tamanho, odor e a composição do néctar. Concluíu-se, então, que era, e que é ainda necessário, até a presente data, integrar os estudos de defesas de plantas com aquele sobre polinização, para o avanço dos estudos em biologia vegetal, especialmente no contexto entre a defesa e o crescimento/reprodução das plantas.

Um experimento de campo foi então estabelecido como o pontapé inicial deste trabalho de tese. O principal objetivo deste experimento foi avaliar os efeitos globais do dano e da resposta induzida por *P. brassicae* na reprodução da planta hospedeira. As respostas da planta ao ataque do herbívoro foram investigadas desde a deposição dos ovos e ao longo de todo o desenvolvimento das lagartas. Neste trabalho de campo, os herbívoros que foram deliberadamente introduzidos, foram expostos a predadores e parasitoides naturalmente presentes no campo, e a dispersão e a mortalidade das lagartas durante o experimento foram registradas. Além disso, foram quantificados os efeitos da infestação pelo especialista *P. brassicae*: (1) no comportamento do polinizador; (2) na emissão de compostos orgânicos voláteis e (3) no número de sementes e no momento em que as sementes começaram a ser produzidas pelas plantas. Notavelmente, observou-se que as plantas infestadas com *P. brassicae* tiveram a produção de sementes aceleradas, em resposta aos ovos depositados pelas borboletas. As lagartas se alimentam de flores, mas não das sementes. Deste modo, as plantas ao acelerarem a produção de sementes, impediram o consumo de flores e se defenderam efetivamente contra esse herbívoro voraz. No geral, as interações com polinizadores generalistas foram mantidas ao longo de todo o ciclo reprodutivo, e as plantas infestadas produziram tantas ou mais sementes, quanto às plantas utilizadas como controle. Insetos herbívoros podem, no entanto, influenciar a visitação de agentes polinizadores de forma diferente, quando determinado a nível da espécie, e também dependendo se foram as folhas, ou as flores, atacadas pelos herbívoros. Assim, nos capítulos 4 e 5 foi investigado como as respostas das plantas induzidas pelo herbívoro podem levar a mudanças no comportamento dos polinizadores.

No Capítulo 4, os efeitos sistêmicos do dano causado pelo herbívoro às folhas, e quantificado nas flores, foram investigados. Efeitos do dano causado por folívoros − herbívoros que se alimentam de folhas − na composição do néctar e nas características das flores utilizadas pelos polinizadores foram abordados, considerando o papel da química defensiva contra herbívoros no comportamento dos polinizadores. A visitação das flores por agentes polinizadores é influenciada pela qualidade e quantidade das recompensas oferecidas pelas flores − como, por exemplo, o néctar floral − e por aspectos morfológicos e composição química das flores que podem ser associados com o valor de tal recompensa. Portanto, o objetivo deste estudo neste capítulo foi inferir se as mudanças nos odores típicos utilizados pelos polinizadores na escolha das flores podem estar associadas às mudanças legítimas na quantidade e qualidade do néctar oferecido pelas flores da mostarda. O dano causado por folívoros influenciaram tanto a emissão de compostos orgânicos voláteis pelas flores quanto o teor de açúcar presente no néctar. Após o dano às folhas causado pelos herbívoros, a emissão de voláteis pelas flores foi alterada, mas não pelas folhas. Interessantemente, a emissão de compostos orgânicos voláteis pelas folhas não diferiu significativamente entre as plantas infestadas e não infestadas. A frequência das visitas nas flores pelos polinizadores, de maneira geral, não foi influenciada pelos folívoros. Entretanto, as abelhas e borboletas passaram menos tempo visitando flores de plantas infestadas com inseto herbívoro comparado as flores das plantas controle, e de fato, o néctar produzido pelas plantas infestadas é mais doce.

No capítulo 5, os mecanismos através dos quais as respostas induzidas por herbívoros por plantas podem afetar o comportamento dos polinizadores foram investigados. Este capítulo aborda não somente respostas induzida pelos herbívoros, mas também as respostas das plantas a polinização. As plantas podem responder às atividades de polinizadores e herbívoros, e as mesmas classes de metabólitos secundários de plantas associadas a respostas induzidas pelos herbívoros pode também estar associada com a atração de agentes polinizadores. Neste capítulo, foi investigado como as respostas das plantas a polinização e ao ataque dos herbívoros podem afetar o comportamento dos polinizadores, quando as lagartas se alimentam das folhas ou das flores da mostarda. Além disso, este capítulo examina o modo como estas plantas respondem a polinização e ao dano causado pelos herbívoros em termos de produção de compostos orgânicos voláteis e não voláteis. E em última análise, o objetivo foi investigar se as respostas das plantas aos herbívoros interferem com respostas a polinização. Os resultados mostram que os adultos usam atributos diferentes quando selecionam a planta para deposição de ovos ou como fonte de néctar. Foi observado de que o sirfideo *Episyrphus balteatus* (De Geer) visitou preferencialmente flores novas ou recentemente abertas, e a polinização em si, não influenciou o comportamento desse inseto o qual coleta preferencialmente pólen das flores da mostada. A planta *B. nigra* respondeu a polinização e ao ataque dos herbívoros com alterações na composição dos compostos voláteis e não voláteis das flores; isto pode potencialmente influenciar a associação da coloração-odor explorada pelos polinizadores quando estão selecionando flores. A natureza sistémica da resposta das plantas induzida por herbívoros pode também interferir com as respostas locais à polinização, e estas são importantes na otimização da reprodução na planta. Os resultados dos capítulos 4 e 5 são discutidos no contexto do conflito entre a defesa da planta e atração de agentes polinizadores, e concluiu-se de que tanto os herbívoros quanto os polinizadores induziram alterações fenotípicas importantes nas flores da mostarda. Além disso, diferentes espécies de agentes polinizadores podem ser diferencialmente afetados por respostas induzidas por herbívoros, dependendo, por exemplo, do tipo de recompensa que eles estão buscando. Portanto, a resposta das plantas à polinização e aos herbívoros devem ser abordadas de forma integrada, pois na natureza, as plantas são expostas, ao mesmo tempo, à herbívoros e polinizadores, e na maioria das vezes, são simultaneamente expostas a várias espécies de polinizadores.

No Capítulo 6, o ponto de vista do herbívoro foi tomado para investigar o motivo pelo qual as lagartas de *P. brassicae* se alimentam das flores. A sobrevivência de insetos herbívoros é normalmente limitada pela capacidade da escolha de alimentos e riscos impostos pelos predadores. Neste capítulo foi investigado se as flores da mostarda podem fornecer às lagartas refúgio quanto aos seus inimigos naturais, e quais seriam às consequências para a sobrevivência de *P. brassicae* quando se alimentam das flores. Foram realizados experimentos no campo e em casa de vegetação para investigar como as borboletas selecionam plantas para deposição de ovos, e as subsequentes interações das lagartas com seus parasitoides e predadores em diferentes partes da planta. Os resultados mostram que, ao moverem-se para as flores, as lagartas escaparam do parasitóide. As flores são também nutricionalmente superiores quando comparadas às folhas, e as lagartas desenvolvem-se mais rapidamente quando alimentam-se de flores. No entanto, no campo, as lagartas em estágio larval final foram intensamente predadas por vespas, independentemente de estarem posicionadas nas folhas ou flores da mostarda. Os resultados deste capítulo são discutidos no contexto das teorias da amplitude da dieta e espaço limitado ou inacessível ao inimigo natural, e em termos da pressão exercida pela seleção natural, e restrições à sobrevivência do herbívoro na natureza. Foi concluído de que a preferência por flores por *P. brassicae* pode ser provavelmente relacionada às vantagens nutricionais e à redução do risco de parasitismo pelo parasitoide especialista quando se alimentam de flores, do que ao risco de serem atacados por predadores generalistas. De maneira geral, em *B. nigra*, insetos carnívoros são determinantes no controle da população de lagartas de *P. brassicae*. Em dois anos consecutivos de experimentos de campo, foi observado que os insetos carnívoros podem matar até 95% das lagartas de *P. brassicae* presentes em *B. nigra*. Assim, no capítulo 7 foi investigado o papel dos insetos carnívoros como componente da estratégia de defesa da planta, e como *B. nigra* aloca os recursos primários após o ataque dos herbívoros. O objetivo geral foi investigar como *B. nigra* equilibra os investimentos em reprodução com aqueles em estratégias defensivas contra *P. brassicae*. Os dados obtidos mostram que as plantas responderam aos ovos depositados e ao dano causado pelas lagartas alocando recursos para a reprodução. As plantas puderam também compensar a perda causada pelos herbívoros em termos de produção de sementes quando as interações com os carnívoros foram mantidas. No entanto, na ausência de insetos carnívoros, *B. nigra* não pode compensar os danos causados pelas lagartas e produziram menos sementes do que as plantas controle. Estes dados suportam a importância de carnívoros como componentes de estratégia de defesa da planta.

Os dados apresentados nesta tese de doutorado contribuem para a compreensão de como as plantas no estado reprodutivo respondem ao ataque dos herbívoros e quais as consequências para a sobrevivência da planta e do herbívoro. Após ataque por herbívoros especialistas, as plantas da mostarda mantem interações com insetos polinizadores e carnívoros, e assim têm sua capacidade reprodutiva sustentada. Este trabalho revelou alguns dos mecanismos que permitem que esta planta anual equilibre investimentos em defesa e reprodução, incluindo mecanismos de resistência e realocação de recursos primários pelas plantas. Ressalto que, para entender mais profundamente como as estratégias de defesa das plantas evoluíram, é necessário integrar estudos envolvendo interações planta-herbívoro e interações planta-polinizador. Em última análise, esses estudos devem ser conduzidos até que a produção de

sementes possa ser quantificada, porque só assim conclusões, em termos evolutivos podem ser geradas. Os resultados deste trabalho, juntamente com estudos recentemente publicados por outros, constituem os primeiros passos nessa direção.

Résumé & publications

Dani Lucas-Barbosa has a bachelor degree in chemistry; this degree was obtained at the Federal University of Rio de Janeiro, in Rio de Janeiro/Brazil, where she grew up. Subsequently, Dani worked both in industry and research, and the experience with research in the south of Brazil and in Italy lighted up her interest in chemical ecology. In 2008, Dani came to Wageningen to follow a master program in plant sciences, and is now specialized in entomology. After obtaining the MSc degree in early 2010, Dani stayed at the Laboratory of Entomology at Wageningen University to pursue a PhD degree. Dani is interested in ecological and evolutionary consequences of induced defences to herbivorous insects, particularly when regarding plant-mediated interactions with carnivorous and pollinating insects. Her contribution so far to this field of research is found in this book that is lying in your hands.

In peer-reviewed journals

Lins, J. C., Jr., J. J. A. van Loon, V. H. P. Bueno, **D. Lucas-Barbosa**, M. Dicke, and J. C. van Lenteren. **2014**. Response of the zoophytophagous predators *Macrolophus pygmaeus* and *Nesidiocoris tenuis* to volatiles of uninfested plants and to plants infested by prey or conspecifics. Biocontrol 59: 707-718.

Lucas-Barbosa, D., E. H. Poelman, Y. Aartsma, T. A. L. Snoeren, J. J. A. van Loon, and M. Dicke. **2014**. Caught between parasitoids and predators - survival of a specialist herbivore on leaves and flowers of mustard plants. Journal of Chemical Ecology 40:621-631. **(chapter 6 in this thesis)**

Bruinsma, M., **D. Lucas-Barbosa**, C. J. M. ten Broeke, N. M. van Dam, T. A. van Beek, M. Dicke, and J. J. A. van Loon. **2014**. Folivory affects composition of nectar, floral odor and modifies pollinator behavior. Journal of Chemical Ecology 40:39-49. **(chapter 4 in this thesis)**

Lucas-Barbosa, D., J. J. A. van Loon, R. Gols, T. A. van Beek, and M. Dicke. **2013**. Reproductive escape: annual plant responds to butterfly eggs by accelerating seed production. Functional Ecology 27:245-254. **(chapter 3 in this thesis)**

Pashalidou, F. G., **D. Lucas-Barbosa**, J. J. A. van Loon, M. Dicke, and N. E. Fatouros. **2013**. Phenotypic plasticity of plant response to herbivore eggs: effects on resistance to caterpillars and plant development. Ecology 94:702-713.

Fatouros, N. E., **D. Lucas-Barbosa**, B. T. Weldegergis, F. G. Pashalidou, J. J. A. van Loon, M. Dicke, J. A. Harvey, R. Gols, and M. E. Huigens. **2012**. Plant volatiles induced by herbivore egg deposition Affect insects of different trophic levels. PLoS ONE 7: e43607.

Lucas-Barbosa, D., J. J. A. van Loon, & M. Dicke. **2011**. The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. Phytochemistry 72:1647-1654. **(chapter 2 in this thesis)**.

Other publications

Lucas-Barbosa, D. and M. Dicke. 2013. Sociale wespen zijn geweldige roofdieren, Dierplagen Informatie, 16(3): 4-7

Submitted or to be submitted for publication

Lucas-Barbosa, D., P. Sun, A. Hakman, T. A. van Beek, J. J. A. van Loon, & M. Dicke. Changing colours and odours: how plant responses to pollination and herbivory affect the behaviour of flower visitors. **(chapter 5 in this thesis)**

Lucas-Barbosa, D., M. Dicke, T. Kranenburg, Y. Aartsma, T. A. van Beek, M. E. Huigens, & J. J. A. van Loon. Seed set of mustard plants is compromised in the absence of natural enemies of herbivores. **(chapter 7 in this thesis)**

Barragán-Fonseca, K., J. J. A. van Loon, M. Dicke, & **D. Lucas-Barbosa**. Flower colours and odours: exploring cues used by insect pollinators in two Brassicaceous plants.

Education statement

Education Statement of the Graduate School

Experimental Plant Sciences

Issued to:	**Dani Lucas-Barbosa**
Date:	**13 March 2015**
Group:	**Laboratory of Entomology**
University:	**Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Trade-off between defence and reproduction in Brassica nigra - PhD meeting	Dec 2010
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
The effects of herbivore-induced plant volatiles on interactions between insects and flowering plants, Phytochemistry 72, 2011, 1647-1654	Sep-Oct 2010
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD Student day, Utrecht University	Jun 01, 2010
EPS PhD Student day, University of Amsterdam	Nov 30, 2012
► **EPS theme symposia**	
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 03, 2011
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Wageningen University	Feb 10, 2012
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 24, 2013
EPS theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 25, 2014
► **NWO Lunteren days and other National Platforms**	
Netherlands Annual Ecology Meeting, Lunteren (NL)	Feb 2011, and Feb 2012
► **Seminars (series), workshops and symposia**	
ExPectationS (EPS Career day)	Nov 19, 2010
Workshop on plant-insect interactions	2011-2014

	Local monthly seminars at the Laboratory of Entomology	2010-2014
	Yearly Entomology Research Exchange Meeting	2011-2014
	Annual Meeting of the Netherlands Entomological Society	2011-2014
►	**Seminar plus**	
►	**International symposia and congresses**	
	14th International Symposium on Insect-Plant Interactions (SIP-14) 2011 , Wageningen, NL	Aug 13-17, 2011
	Gordon Research Conference on Plant Volatiles, Ventura, CA, USA	Jan 29-Feb 03, 2012
	International Symposium: Future of Butterflies in Europe III, Wageningen, NL	Mar 29-31, 2012
	Gordon Research Conference on Plant Volatiles, Ventura, CA, USA	Jan 26-31, 2014
	15th International Symposium on Insect-Plant Relationships, Schwitzerland	Aug 17-22, 2014
	British and French Ecological Societies- joint annual meeting, Lille, France	Dec 09-12, 2014
►	**Presentations**	
	14th International Symposium on Insect-Plant Relationships, Wageningen - poster	Aug 13-17, 2011
	Gordon Research Conference on Plant Volatiles - poster	Jan 29-Feb 03, 2012
	Netherlands Annual Ecology Meeting - oral	Feb 2012
	International Symposium: Future of Butterflies in Europe III - oral	Mar 29-31, 2012
	7th Workshop on plant-insect interactions - oral	Nov 28, 2012
	Yearly Entomology Research Exchange Meeting - oral	2013
	EuroVOL summer school on plant volatiles - poster	Sep 09-12, 2013
	Entomology PhD excursion Switerzerland - oral	Oct 28-Nov 01, 2013
	Gordon Research Conference on Plant Volatiles - oral	Jan 26-31, 2014
	15th International Symposium on Insect-Plant Relationships, Neuchâtel - oral and poster	Aug 17-22, 2014
	Annual Meeting of the Netherlands Entomological Society - oral	Dec 2014
	British and French Ecological Societies- joint annual meeting - oral	Dec 09-12, 2014
►	**IAB interview**	
	Meeting with a member of the International Advisory Board (IAB)	Nov 15, 2012
►	**Excursions**	
	Entomology PhD excursion Switerzerland	Oct 28-Nov 01, 2013
	Subtotal Scientific Exposure	*28.4 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Design of Experiments (WIAS)	Dec 08-10, 2010
	Basic Statistics (PE&RC)	Dec 14-16, 21-22, 2010
	Generalized linear models (PE&RC)	Jun 13-14, 2013

	Mixed linear models (PE&RC)	Jun 20-21, 2013
	EuroVOL summer school on plant volatiles	Sep 09-12, 2013
►	**Journal club**	
	PhD journal club Entomology	2010-2014
	Insect-Plant Interactions discussion group at Entomology	2010-2014
►	**Individual research training**	
	Subtotal In-Depth Studies	*7.7 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Techniques for Writing and Presenting a Scientific Paper	Oct 18-21, 2011
	Advanced course Guide to Scientific Artwork	Nov 07-08, 2011
	Lecturing	Jun 21, 2012
	MS Access	Nov 2012
	Reviewing a Scientfic Paper	Apr 23, 2013
	Adobe InDesign	Nov 06, 2013
►	**Organisation of PhD students day, course or conference**	
	Organising 8th Workshop Plant-Insect interactions	2013
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*4.4 credits**

TOTAL NUMBER OF CREDIT POINTS*	**48.0**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS credits	

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

Appendix A

The effects of herbivore-induced plant volatiles on flower visiting insects - a review

Dani Lucas-Barbosa, Joop J. A. van Loon, & Marcel Dicke

Phytochemistry 2011, 72, 1647-1654

Table A1a Constitutive and inducible volatile organic compounds (VOCs) of a selection of brassicaceous plants. VOCs are represented by: constitutive VOCs emitted from leaves (CL); inducible VOCs emitted from leaves (IL); constitutive VOCs emitted from flowers (CF); inducible VOCs emitted from flowers (IF). Stereochemistry is not reported in some of the studies, and has therefore not been indicated.

	A. lyrata[1]				***A. thaliana***[2]				***B. napus***[3]			
volatile organic compounds	CL	IL	CF	IF	CL	IL	CF	IF	CL	IL	CF	IF
aliphatics												
decanal			■									
1-hexanol											■	
3-hexen-1-ol					■						■	
3-hexenyl acetate					■						■	
nonanal			■									
1-nonanol					■							
1-octen-3-ol					■							
1-pentanol					■	■						
2-penten-1-ol					■							
benzenoids/phenylpropanoids												
acetophenone			■									
benzaldehyde			■								■	
ethyl salicylate					■	■						
methyl benzoate					■	■					■	
methyl salicylate		■			■	■						
phenyl acetaldehyde			■								■	
2-phenyl ethanol											■	
homoterpenes												
4,8-dimethyl-1,3,7-nonatriene	■	■										
4,8,12-trimethyl-1,3,7,11-tridecatetraene		■			■	■	■					
monoterpenes												
3-carene							■				■	
1,8-cineole									■		■	
limonene							■		■		■	
linalool					■	■	■				■	
β-myrcene					■		■		■		■	
β-ocimene					■		■				■	
α-pinene					■		■		■		■	
β-pinene									■		■	
sabinene							■		■		■	
α-thujene							■		■		■	
sesquiterpenes												
β-caryophyllene		■					■					
caryophyllene oxide							■					
β-chamigrene							■					
3-cyclosativene	■	■										
α-farnesene					■	■	■				■	
β-farnesene												
α-humulene		■										
nerolidol		■										
thujopsene							■					
nitrogen-containing compounds												
benzyl cyanide											■	
indole					■	■					■	

[1] *Arabidopsis lyrata* (Abel *et al.* 2009). Leaf VOCs induced by *Plutella xylostella* caterpillars feeding on leaves. No statistical evidence for induction provided. [2] *Arabidopsis thaliana* (Tholl *et al.* 2005, Snoeren *et al.* 2010). Leaf VOCs induced by *Pieris rapae* caterpillars feeding on leaves. [3] *Brassica napus* (Jakobsen *et al.* 1994, Jonsson *et al.* 2005). Data for inducible VOCs not found in the literature.

Table A1b Constitutive and inducible VOCs of a selection of solanaceous plants. VOCs are represented by: constitutive VOCs emitted from leaves (CL); inducible VOCs emitted from leaves (IL); constitutive VOCs emitted from flowers (CF); inducible VOCs emitted from flowers (IF). Stereochemistry is not reported in some of the studies, and has therefore not been indicated.

	N. attenuata[4]				***N. suaveolens***[5]				***S. peruvianum***[6]			
volatile organic compounds	CL	IL	CF	IF	CL	IL	CF	IF	CL	IL	CF	IF
aliphatics												
geranylacetone									🟩		🟨	🟥
2-ethyl-1-hexenal			🟨									
3-hexen-1-ol	🟩	🟩										
3-hexenyl acetate	🟩	🟩				🟩						
3-hexenyl butanoate	🟩	🟩				🟩						
nonanal			🟨									
decanal			🟨									
benzenoids/phenylpropanoids												
acetophenone			🟨									
benzaldehyde			🟨									
benzyl acetone			🟨									
benzyl alcohol			🟨				🟨					
benzyl benzoate							🟨					
benzyl salicylate							🟨					
cinnamaldehyde							🟨					
cinnamyl alcohol							🟨					
p-cresol							🟨					
3,5-dimethoxybenzaldehyde									🟩		🟨	🟥
3,5-dimethoxytoluene									🟩		🟨	
methyl benzoate							🟨					
methyl cinnamate							🟨					
methyl salicylate		🟩					🟨		🟩		🟨	
2-phenylethyl alcohol							🟨					
2-phenylethyl benzoate							🟨					
monoterpenes												
1,8-cineole							🟨					
limonene									🟩		🟨	
linalool	🟩					🟩			🟩		🟨	
β-myrcene									🟩		🟨	
β-ocimene	🟩	🟩				🟩			🟩		🟨	
β-phellandrene									🟩		🟨	
α-pinene									🟩		🟨	
β-pinene									🟩		🟨	
terpineol	🟩											
sesquiterpenes												
α-bergamotene	🟩	🟩				🟩						
β-caryophyllene												
α-farnesene						🟩	🟨					
β-farnesene	🟩	🟩				🟩						
cyclic compounds												
cis-jasmone									🟩		🟨	
nitrogen-containing compounds												
indole							🟨					
methyl nicotinate							🟨					
nicotine	🟩	🟩	🟨	🟥								

[4] *Nicotiana attenuata* (Euler & Baldwin 1996, Kessler & Baldwin 2001). Leaf VOCs induced by *Manduca quinquemaculata* caterpillars feeding on leaves. Flower VOCs induced by *Manduca sexta* caterpillars feeding on flowers. [5] *Nicotiana suaveolens* (Effmert *et al.* 2008). Leaf VOCs induced by *M. sexta* feeding on leaves. Data for constitutive volatiles emitted by leaves not provided. [6] *Solanum peruvianum* (Kessler & Halitschke 2009). Leaf and flower VOCs induced by *M. sexta* feeding on leaves.

Table A1c Constitutive and inducible VOCs of other studied plants, of which HIPVs from flowers have been investigated. VOCs are represented by: constitutive VOCs emitted from leaves (CL); inducible VOCs emitted from leaves (IL); constitutive VOCs emitted from flowers (CF); inducible VOCs emitted from flowers (IF). Stereochemistry is not reported in some of the studies, and has therefore not been indicated.

	C. pepo[7]				***G. hirsutum***[8]				***P. sativa***[9]			
volatile organic compounds	CL	IL	CF	IF	CL	IL	CF	IF	CL	IL	CF	IF
aliphatics												
butyl butanoate											🟨	🟥
geranylacetone			🟨	🟥								
2-hexenal					🟩	🟩	🟨					
3-hexenal					🟩		🟨					
3-hexen-1-ol					🟩	🟩	🟨					
3-hexenyl acetate					🟩	🟩	🟨	🟥				
3-hexenyl butanoate						🟩		🟥				
3-hexenyl isobutanoate								🟥				
3-hexenyl-2-methylbutanoate						🟩		🟥				
hexyl acetate								🟥				
6-methyl-5-hepten-2-one			🟨									
1-octanol											🟨	
octyl acetate											🟨	🟥
octyl butanoate											🟨	🟥
benzenoids/phenylpropanoids												
p-anisaldehyde			🟨	🟥								
1,4-dimethoxybenzene			🟨									
1,2,4-trimethoxybenzene			🟨									
myristicin											🟨	🟥
homoterpenes												
4,8-dimethyl-1,3,7-nonatriene			🟨	🟥	🟩	🟩		🟥				
4,8,12-trimethyl-1,3,7,11-tridecatetraene					🟩	🟩		🟥				
monoterpenes												
limonene					🟩		🟨					
linalool					🟩	🟩	🟨	🟥				
β-myrcene					🟩		🟨					
α-ocimene											🟨	🟥
β-ocimene					🟩	🟩	🟨	🟥			🟨	🟥
α-pinene					🟩		🟨					
β-pinene					🟩		🟨					
sesquiterpenes												
β-bergamotene											🟨	
γ-bisabolene					🟩							
β-caryophyllene							🟨				🟨	🟥
α-farnesene					🟩	🟩		🟥				
β-farnesene					🟩	🟩		🟥			🟨	🟥
germacrene D			🟨	🟥							🟨	🟥
α-gurjunene			🟨									
α-humulene					🟩		🟨					
nerolidol								🟥				
cyclic compounds												
cis-jasmone								🟥				
nitrogen-containing compounds												
indole						🟩		🟥				

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[7] *Cucurbita pepo* subsp. *texana* (Theis *et al.* 2009). Male flower VOCs induced by simulated beetle damage to leaves. [8] *Gossypium hirsutum* (Loughrin *et al.* 1994, Röse & Tumlinson 2004). Leaf VOCs induced by *Spodoptera exigua* caterpillars feeding on leaves. Flower bud VOCs induced by *Helicoverpa zea* caterpillars feeding on buds. [9] *Pastinaca sativa* (Zangerl & Berenbaum 2009). Flower VOCs induced by *Depressaria pastinacella* webworm feeding on flowers.

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Appendix B

Reproductive escape: annual plant responds to butterfly eggs by accelerating seed production

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Teris A. van Beek & Marcel Dicke

Functional Ecology **2013**, 27, 245-254

Table B1. ANOVA repeated measures of time effects of the treatment on behaviour of pollinators for trial 1

Source	**Bumblebees**			**Honey bees**			**Syrphid flies**		
	df	**MS**	***P***	***df***	**MS**	***P***	***df***	**MS**	***P***
Time spent per flower									
Between subjects									
Treatment	1	1.45	0.846	1	9.07	0.603	1	112.76	0.146
Error	22	37.36		28	32.79		23	49.70	
Within subjects									
Time	1	81.53	0.155	1	17.07	0.366	1	39.79	0.200
Treatment x time	1	15.54	0.527	1	18.72	0.344	1	1.44	0.804
Error	22	37.59		28	20.20		23	22.82	
Number of flowers visited									
Between subjects									
Treatment	1	552.00	0.312	1	14.84	0.889	1	7.05	0.757
Error	29	521.32		29	746.44		23	71.79	
Within subjects									
Time	1	8271.77	**0.001**	1	280.14	0.454	1	284.08	0.117
Treatment x time	1	1108.29	0.180	1	165.17	0.565	1	48.48	0.508
Error	29	587.14		29	486.40		23	107.30	
Number of visitors									
Between subjects									
Treatment	1	1.45	0.846	1	0.28	0.670	1	12.09	0.278
Error	22	37.36		28	1.49		37	9.98	
Within subjects									
Time	1	81.53	0.155	1	0.54	0.538	1	22.42	0.124
Treatment x time	1	15.54	0.527	1	4.01	0.101	1	33.44	0.062
Error	22	37.59		28	1.40		37	9.03	

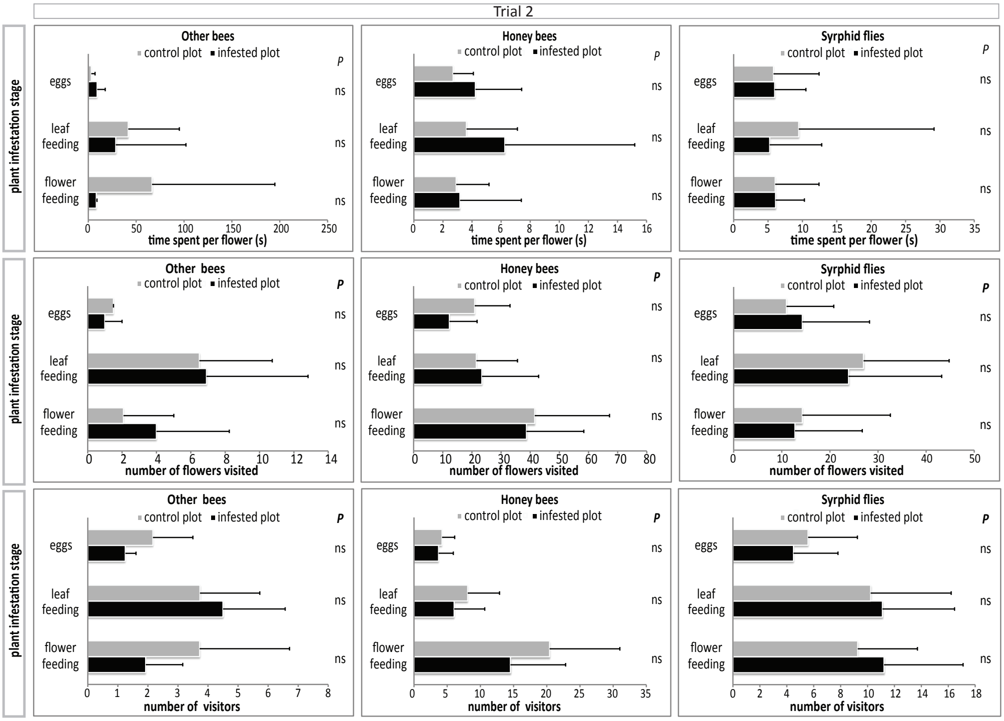

Fig. B1 Time spent per flower (s) (mean + SD), number of flowers visited (mean + SD) and number of flower visitors per plot (mean + SD) to *Pieris brassicae*-infested and control *Brassica nigra* plots, per 10 min of observation. Visitation by bumbles bees, honey bees and syrphid flies to infested and control plots were recorded when plants carried eggs and when most herbivores were feeding on leaves or flowers. Results are presented for trial 2. Pairwise comparison of treatments with independent *t*-test, or Mann-Whitney U-test when data did not follow normal distribution (ns, not significant).

Table B2. ANOVA repeated measures of time effects of the treatment on behaviour of pollinators for trial 2.

Source		**Honey bees**			**Syrphid flies**	
	df	**MS**	***P***	***df***	**MS**	***P***
Time spent per flower						
Between subjects						
Treatment	1	5.04	0.263	1	8.12	0.414
Error	14	3.71		7	10.78	
Within subjects						
Time	2	7.09	0.328	2	21.93	0.159
Treatment x time	2	3.54	0.566	2	9.30	0.432
Error	28	6.11		14	10.43	
Number of flowers visited						
Between subjects						
Treatment	1	270.24	0.256	1	28.85	0.681
Error	14	192.84		9	159.47	
Within subjects						
Time	2	2550.19	**0.020**	2	444.70	0.371
Treatment x time	2	96.82	0.843	2	193.24	0.641
Error	28	561.87		18	424.28	
Number of visitors						
Between subjects						
Treatment	1	42.48	0.423	1	0.30	0.925
Error	21	63.72		16	32.36	
Within subjects						
Time	* 1	2285.96	**0.001**	2	161.56	**0.018**
Treatment x time	1	10.82	0.725	2	46.74	0.281
Error	29	50.02		32	35.40	

Table B3. Volatile compounds of *Brassica nigra* infested at 3 stages during development of *Pieris brassicae* larvae, and control plants of the same stages.

Volatile compounds	egg-control	egg-infested	leaf-control	leaf-infested	flower-control	flower-infested
	Peak area ± SD*	Peak area ± SD	Peak area ± SD	Peak area ± SD	Peak area ± SD	Peak area ± SD
Benzenoids and phenyl propanoids						
Acetophenone	219 ± 215	337 ± 447	141 ± 159	140 ± 74	463 ± 938	123 ± 36
Benzaldehyde	112 ± 56	485 ± 763	132 ± 91	128 ± 57	570 ± 1070	199 ± 101
Benzyl Alcohol	2390 ± 4560	1220 ± 1390	618 ± 914	340 ± 288	608 ± 720	576 ± 536
Methyl salicylate	-†	-	-	-	20 ± 10	ss‡
Monoterpenoids						
β-Myrcene	-	-	-	-	13 ± 2	40 ± 7
1,7-Octadien-3-one, 2-methyl-6-methylene-	-	-	14 ± 14	12 ± 7	69 ± 85	13 ± 7
α-Pinene	-	-	-	-	119 ± 175	ss
Pinocarvone	-	-	-	-	14 ± 9	19 ± 6
Sabinene	-	-	99 ± 112	114 ± 142	ss	31 ± 4
Verbenone	-	-	27 ± 16	ss	48 ± 67	18 ± 2
Homoterpenoids						
4,8-dimethyl-1,3,7-nonatriene -(E)	71 ± 37	278 ± 470	80 ± 51	53 ± 56	211 ± 309	35 ± 9
4,8-dimethyl-1,3,7-nonatriene -(Z)	-	-	30 ± 19	38 ± 30	135 ± 257	13 ± 6
Sesquiterpenoids						
α-Farnesene, (Z/E)-	-	-	18 ± 12	8 ± 4	13 ± 6	ss
Longicyclene	92 ± 179	136 ± 206	10 ± 5	51 ± 98	44 ± 66	9 ± 3
Longifolene	194 ± 383	82 ± 97	9 ± 3	43 ± 83	29 ± 40	61 ± 101
Isobornyl butanoate	377 ± 662	510 ± 872	72 ± 20	60 ± 28	210 ± 356	142 ± 145
Isobornyl propionate	388 ± 442	1120 ± 1910	147 ± 44	130 ± 69	44 ± 718	330 ± 330
7-β-H-Silphiperfol-5-ene	-	-	14 ± 9	47 ± 53	-	-
7-β-H-Silphiperfol-5-ene	ss	17 ± 5	42 ± 40	89 ± 95	-	-
Unknown m/z 206	252 ± 337	212 ± 241	9 ± 4	43 ± 86	32 ± 39	14 ± 8
Fatty acid derivatives						
Decanal	415 ± 602	377 ± 310	73 ± 46	116 ± 66	527 ± 944	157 ± 134
3-Hexen-1-ol, acetate, (Z)-	87 ± 55	440 ± 708	83 ± 97	48 ± 24	-	-
Nonanal	322 ± 510	253 ± 212	47 ± 23	75 ± 45	269 ± 485	173 ± 189
Octanal	39 ± 17	35 ± 31	-	-	152 ± 175	51 ± 10
Total volatile emission	4706± 8055	5502± 7662	1463± 1679	1535± 1305	3989± 6471	2004± 1634

*Values for peak area (mean ± SD) were divided by 10^6. † (-) indicates that compound was not detected in any sample. ‡ (ss) indicates the compound was detected in a single sample.

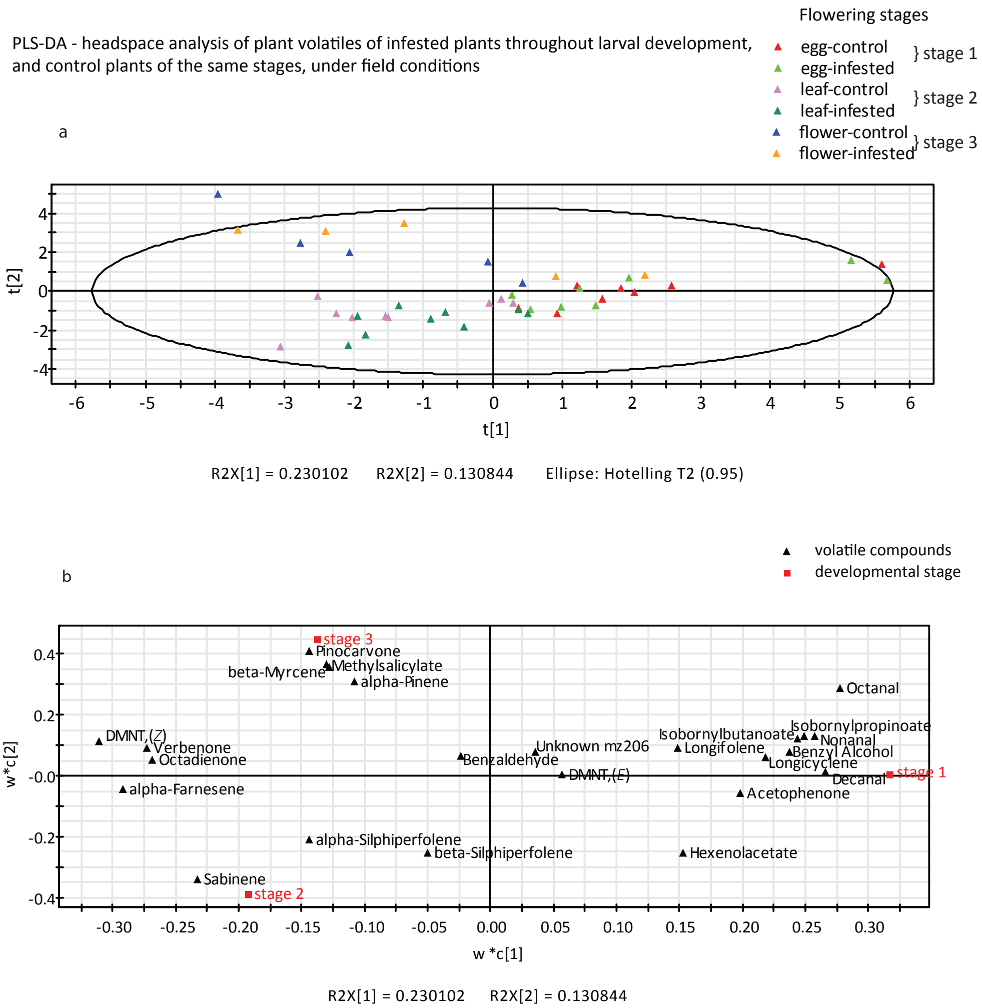

Fig. B2 PLS-DA on volatile compounds collected from *Pieris brassicae*-infested plants during egg-infestation, leaf-feeding phase and flower-feeding phase, and from control plants of the same stages. Projection to Latent Structures Discriminant Analysis (PLS-DA) on the peak area (log-transformed data) of volatile compounds from headspace of *Brassica nigra* plants. a) Grouping pattern of samples according to the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot b) Contribution of each of the volatile compounds to the first two principal components is shown in the loading plot of the PLS-DA components.

Appendix C

Changing colours and odours: how plant responses to pollination and herbivory affect the behaviour of flower visitors

Dani Lucas-Barbosa, Pulu Sun, Anouk Hakman,
Teris A. van Beek, Joop J.A. van Loon & Marcel Dicke

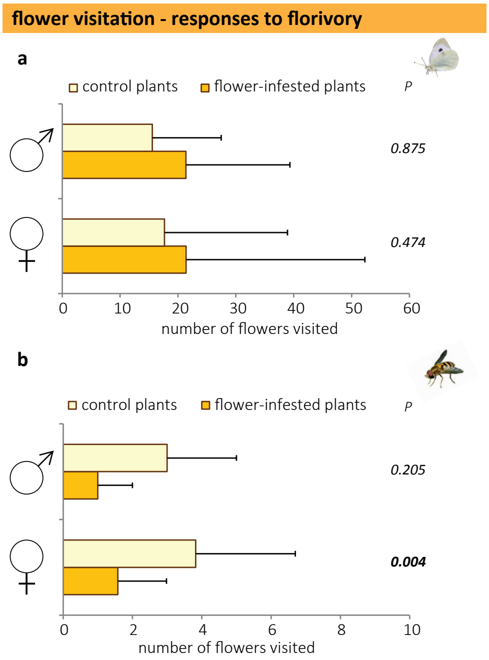

Fig. C1 Number of *Brassica nigra* flowers visited by male and female *Pieris brassicae* butterflies (a) and *Episyrphus balteatus* syrphid flies (b) to flower-infested and non-infested control plants. Experiments were carried out 120 h after *P. brassicae* caterpillars had hatched from the eggs. Pairwise comparison of treatments with generalized linear model with Poisson distribution. At least 30 insects of each sex were tested with 5-8 pairs of plants.

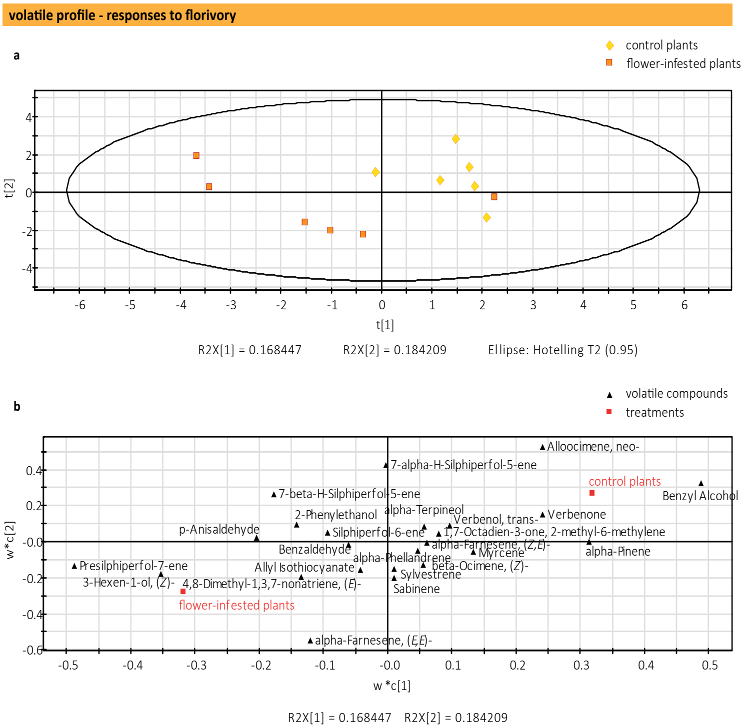

Fig. C2 Projection to Latent Structure Discriminant Analysis (PLS-DA) of volatile compounds collected from flower-infested *Brassica nigra* plants, and from non-infested control plants. Plant volatiles were collected from aerial parts of plants 120 hours after larvae of *Pieris brassicae* had hatched from eggs. PLS-DA on the peak area of volatile compounds from headspace of *B. nigra* plants. a) Grouping pattern of samples according to the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; b) Contribution of individual volatile compounds to the first two principal components is shown in the loading plot of the PLS-DA components.

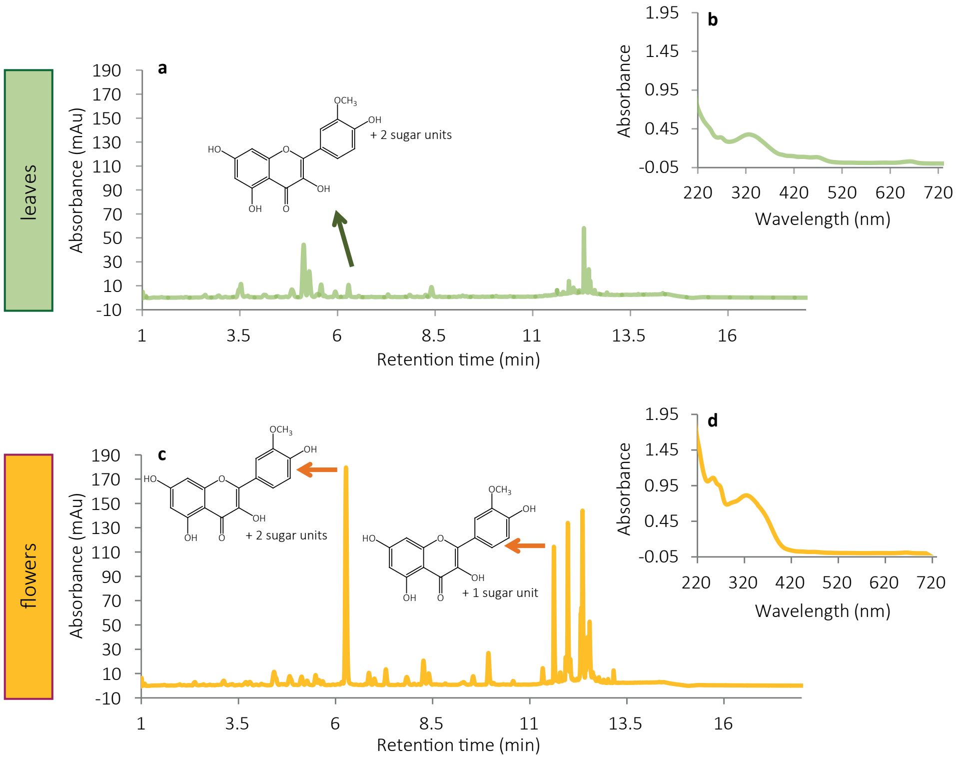

Fig. C3 Typical UHPLC 330 nm profile of extracts of leaves (a) and flowers (b) of *Brassica nigra* plants. Off-line UV-Vis spectra of the crude aqueous methanolic extracts of leaves (b) and flowers (d) are shown from 220 nm to 720 nm. UV-Vis spectra show average profile for 6 samples, in each case.

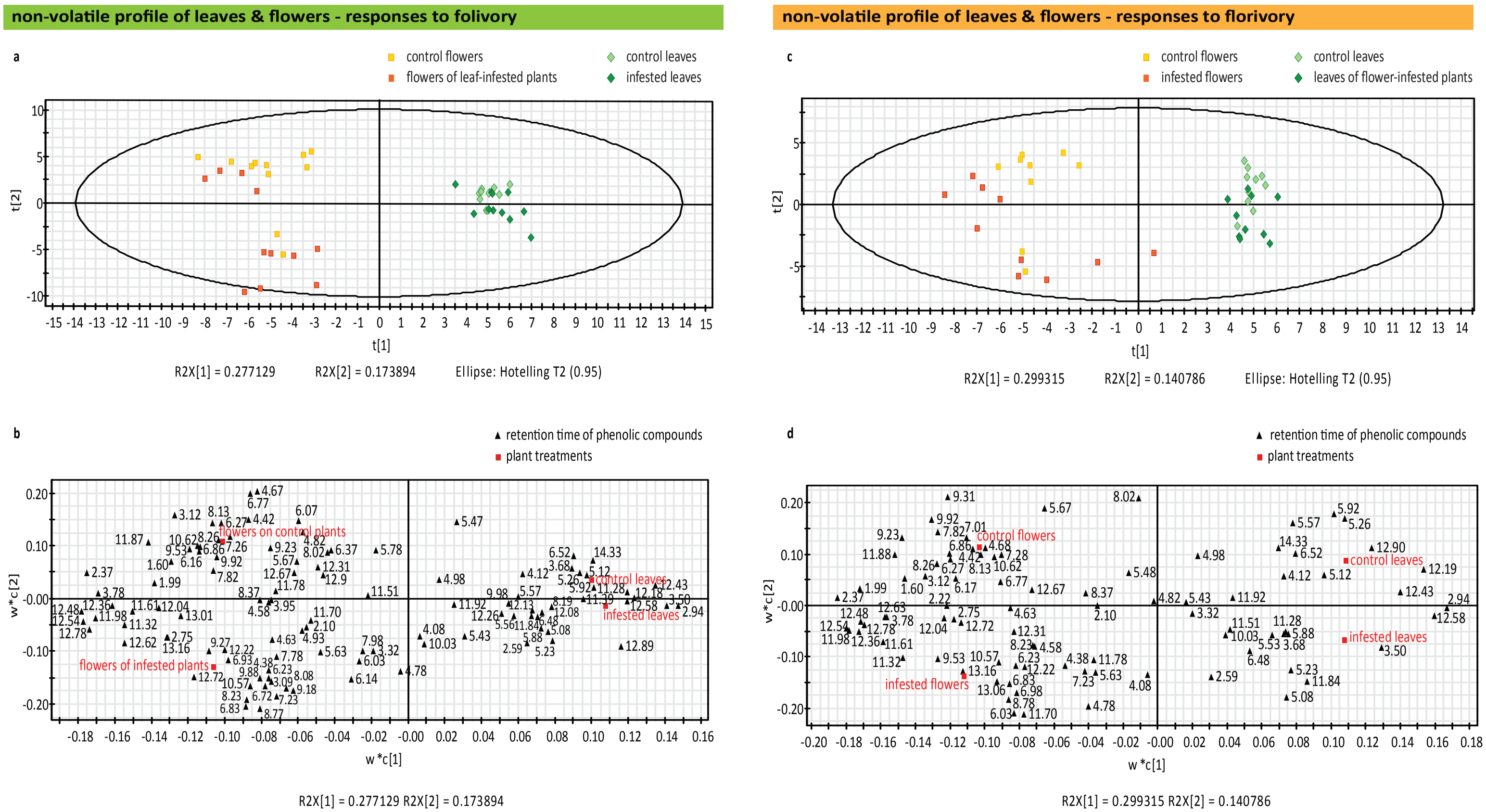

Fig. C4 PLS-DA on non-volatile compounds extracted from leaves and flowers of *Pieris brassicae*-infested plants and control *Brassica nigra* plants of the same stage, at two time points: 24 h and 120 h caterpillars had hatched from eggs. Projection to Latent Structure Discriminant Analysis (PLS-DA) on the peak area of non-volatile compounds from leaves and flowers of *B. nigra* plants. (a and c) Grouping pattern of samples based on the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; (b and d) Loading plot of the PLS-DA components shows the contribution of individual compounds to the first two principal components. Numbers refer to the retention time in the UHPLC profiles.

non-volatile profile of leaves - responses to pollination & folivory

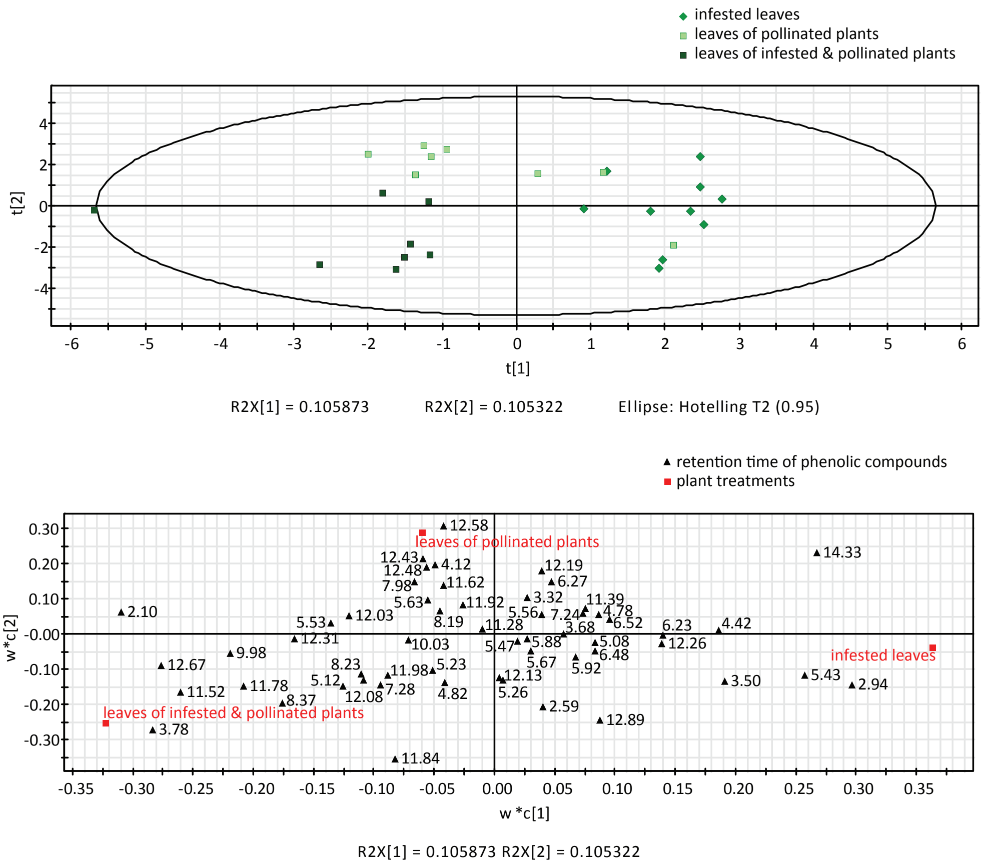

Fig. C5 PLS-DA on non-volatile compounds extracted from leaves of *Pieris brassicae*-infested plants, pollinated plants and of *Brassica nigra* plants that were pollinated and infested with *P. brassicae* caterpillars. Projection to Latent Structure Discriminant Analysis (PLS-DA) on the peak area of phenolic compounds from leaves of *B. nigra* plants. (a) Grouping pattern of three differently treated leaf samples based on the first two principal components, and the Hotelling's T2 ellipse confining the confidence region (95%) of the score plot; (b) Loading plot of the PLS-DA components shows the contribution of individual compounds to the first two principal components. Numbers refer to the retention time in the UHPLC profiles.

non-volatile profile of insect-pollinated & hand-pollinated plants

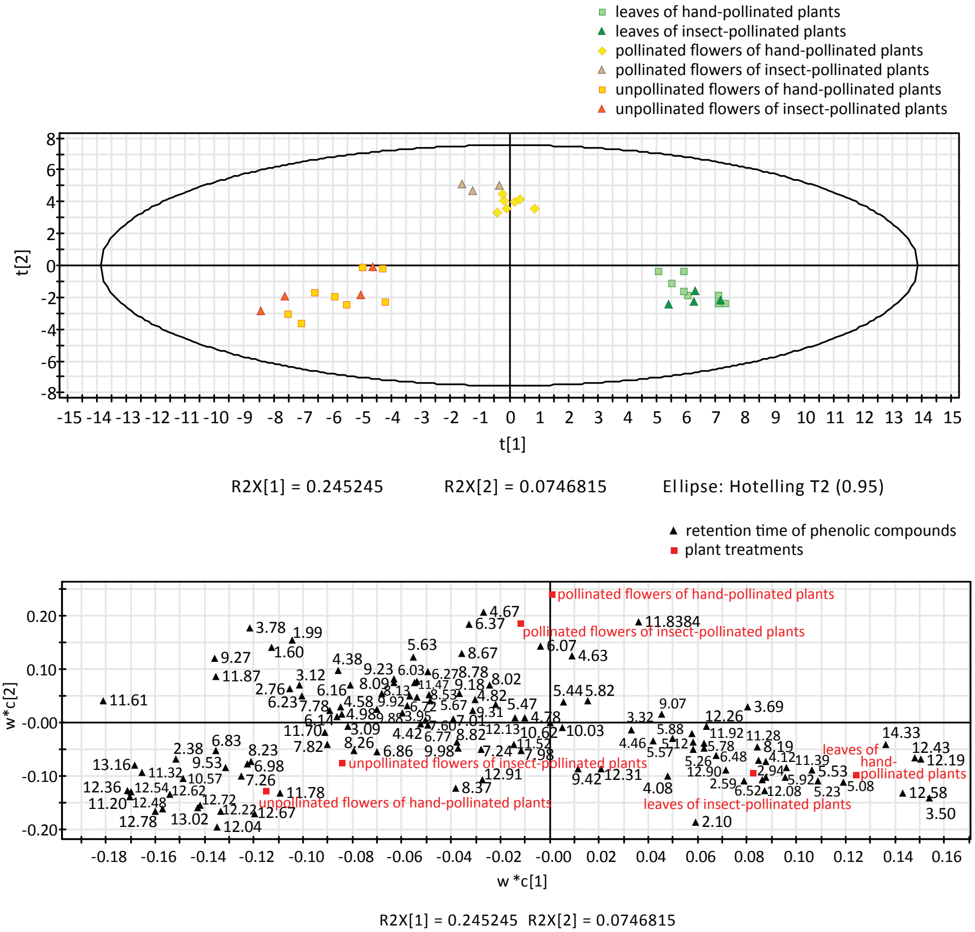

Fig. C6 PLS-DA on non-volatile compounds extracted from pollinated and unpollinated flowers and leaves of hand-pollinated *Brassica nigra* plants and of insect-pollinated plants by male butterflies of *Pieris brassicae*. Projection to Latent Structure Discriminant Analysis (PLS-DA) on the peak area of individual compounds from leaves and flowers of *B. nigra* plants. (a) Grouping pattern of samples based on the first two principal components, and the Hotelling's T2 ellipse confining the confidence region (95%) of the score plot; (b) Loading plot of the PLS-DA components shows the contribution of individual compounds to the first two principal components. Numbers refer to the retention time in the UHPLC profiles.

Table C1 Partial identification of phenolic compounds: retention time (RT), molecular formula, the exact mass and maximum UV-Vis absorbance is shown for compounds identified in leaves and flowers of *Brassica nigra* plants.

compounds	RT (min)	formula	$[M+H]^+/[M-H]^-$	UV-Vis abs (nm)	plant part
Quercetin dihexoside	4.83	$C_{27}H_{30}O_{17}$	−/625.1	–	flowers
Kaempferol trihexoside	5.24	$C_{33}H_{40}O_{21}$	−/771.2	–	leaves/flowers
Isorhamnetin dihexoside	6.77	$C_{28}H_{32}O_{17}$	641.2/639.2	226, 255, 354	leaves/flowers
Quercetin dihexoside	7.30	$C_{27}H_{30}O_{17}$	−/625.1	–	flowers
Kaempferol dihexoside	7.82	$C_{27}H_{30}O_{16}$	−/609.1	–	flowers
Isorhamnetin hexoside	11.70	$C_{22}H_{22}O_{12}$	479.1/477.1	226, 255, 355	flowers

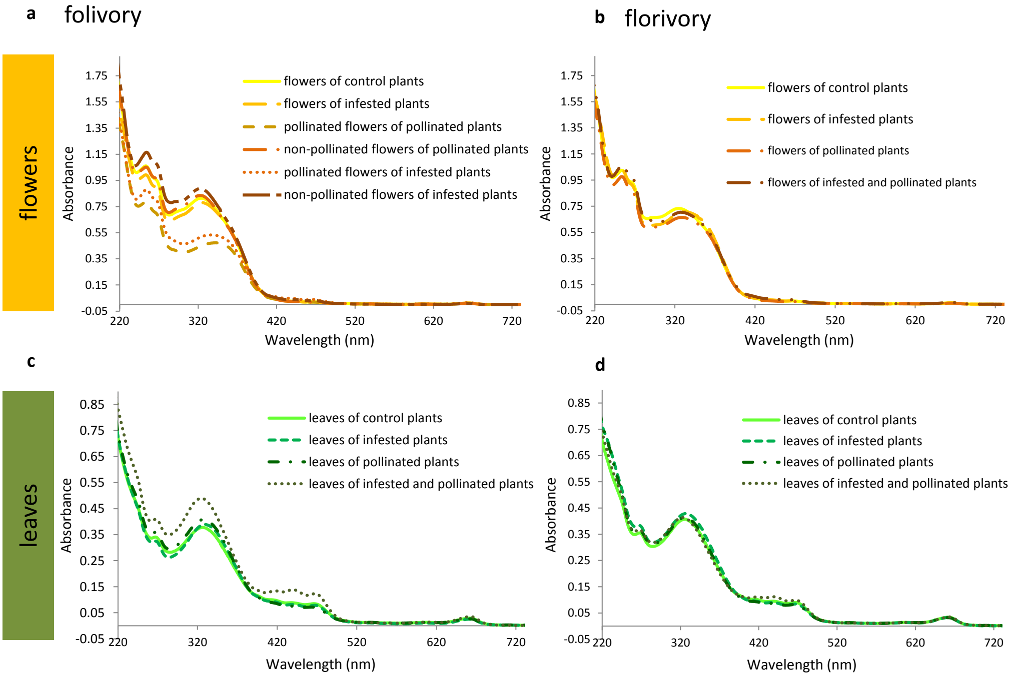

Fig. C7 Ultraviolet-visible (UV-Vis) absorbance spectra of the extracts of leaves and flowers of *Brassica nigra* plants subjected to different treatments. UV-Vis spectra are shown for leaves of control plants, *Pieris brassicae*-infested plants, pollinated plants and of plants that were both pollinated and infested with *P. brassicae* caterpillars at two time points. Flowers (a) and leaves (c) of plants exposed to folivory for 24 h since larvae had hatched from eggs; Flowers (b) and leaves (d) exposed to folivory and florivory for 120 h since larvae had hatched from eggs. Absorbance is shown from 220 nm to 720 nm.

Appendix D

Caught between parasitoids and predators – survival of a specialist herbivore on leaves and flowers of mustard plants

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Tjeerd A.L. Snoeren, Joop J.A. van Loon & Marcel Dicke

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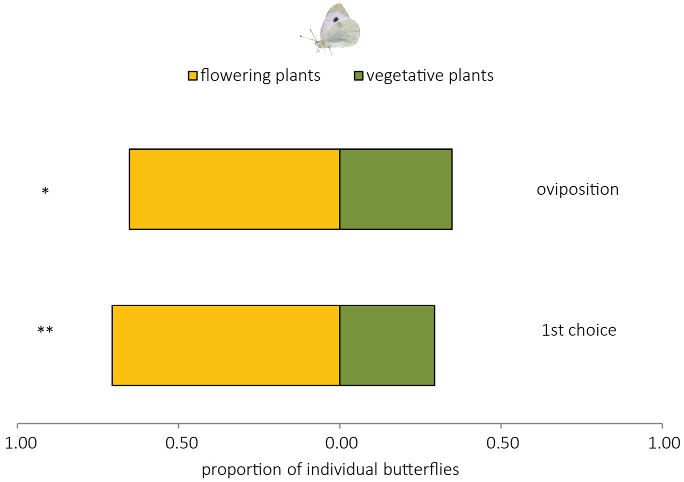

Fig. D1 Proportion of mated female *Pieris brassicae* butterflies that landed first and oviposited on *Brassica nigra* plants in the flowering or vegetative stages. Out of the 52 individual butterflies tested 49 made a choice. Pairwise comparison of treatments with generalized linear model with binomial distribution (α = 0.05; *, $P \leq 0.05$, **, $P \leq 0.01$).

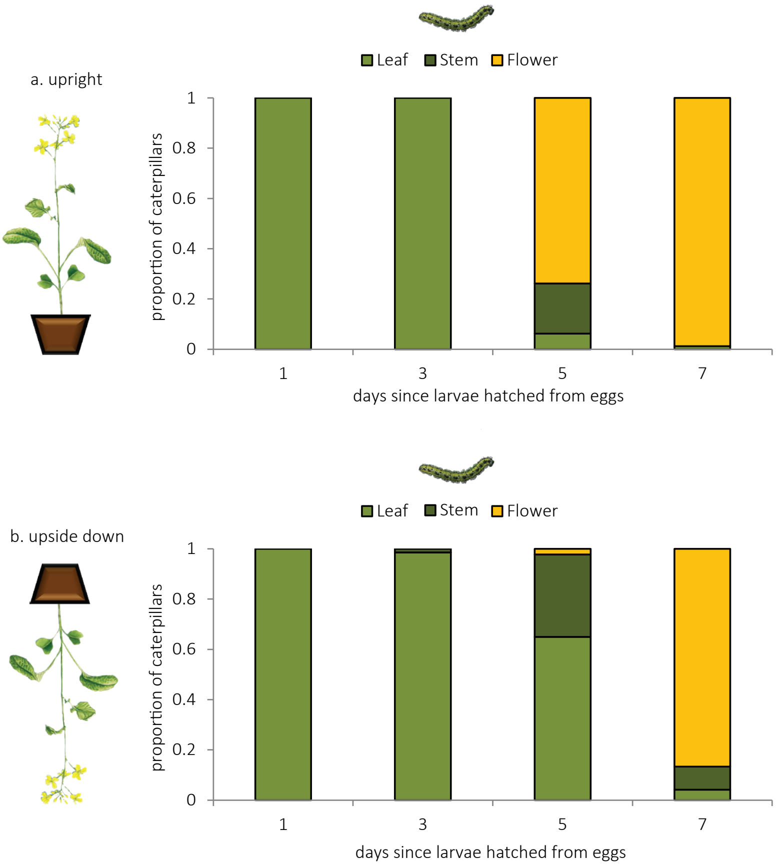

Fig. D2 Proportion of caterpillars found on *Brassica nigra* leaf, stem or flower since larvae hatched from eggs and over time in days, for plants in the upright position (a) and for plants positioned upside down (b). Five plants per treatment were used.

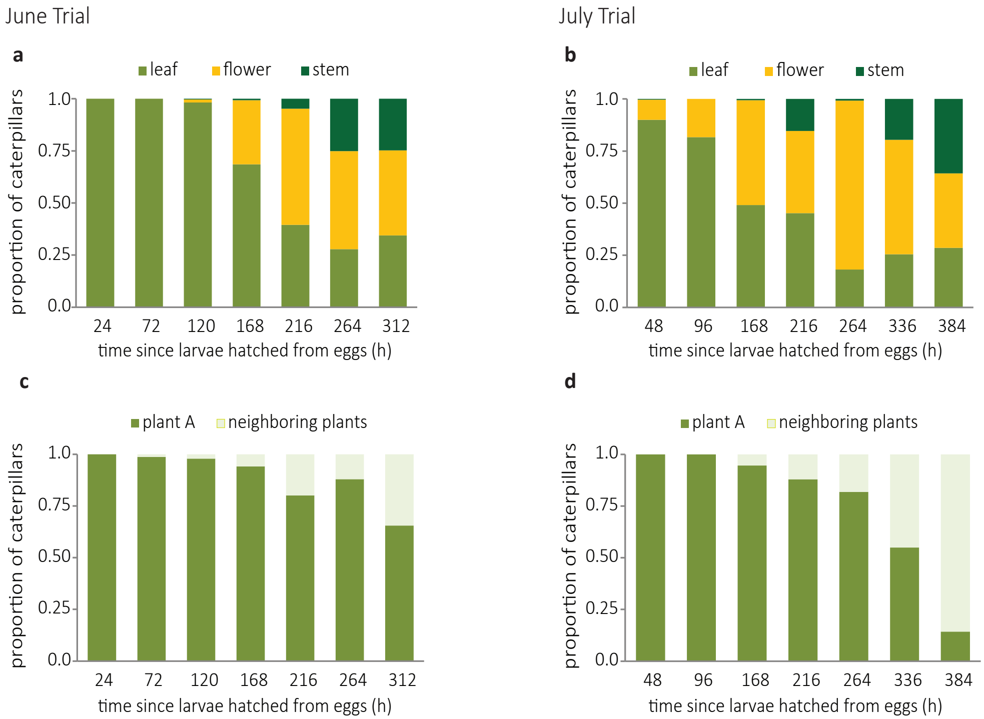

Fig. D3 Dispersal of *Pieris brassicae* caterpillars on plots of *Brassica nigra* plants over time. Proportion of caterpillars found on a leaf, flower or stem of *B. nigra* plants as a function of the time since larvae hatched from eggs, for June (a) and July (b) trials; Proportion of caterpillars found on an infested plant in the centre of the plot or on one of the four neighbouring plants in that plot, since larvae hatched from eggs, for June (c) and July (d) trials.

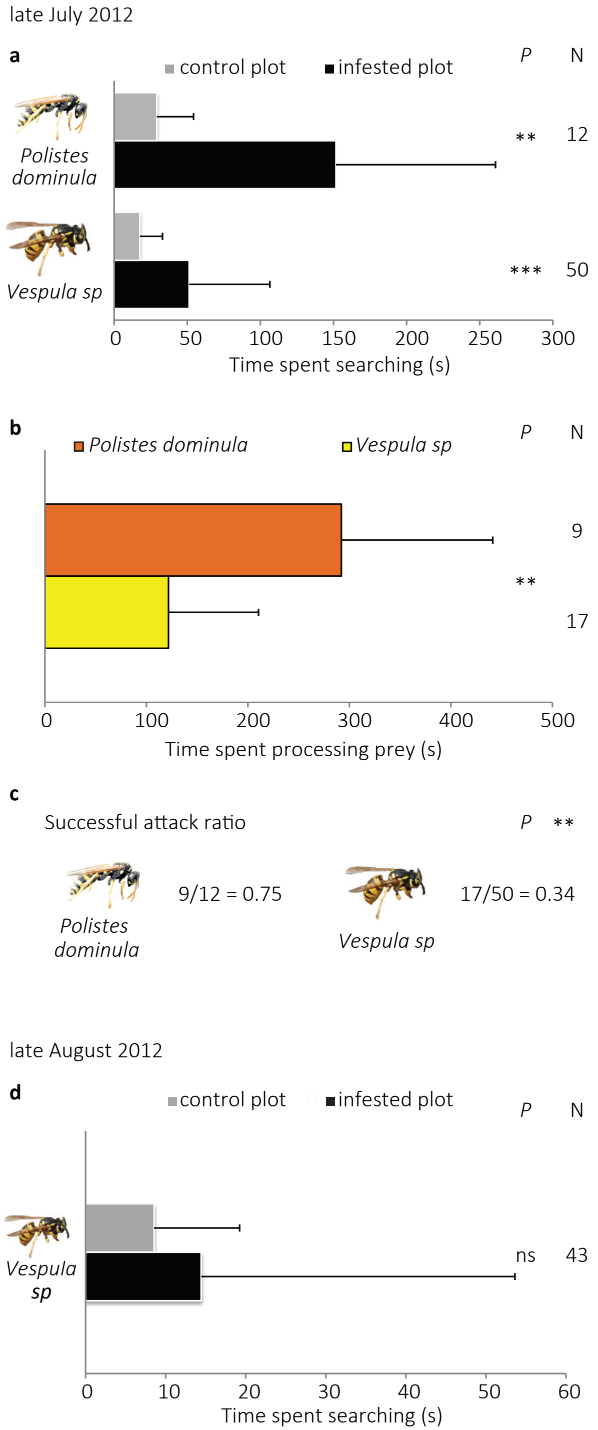

Fig. D4 Foraging behavior of social wasps *Polistes dominula* and *Vespula sp.* on non-infested *Brassica nigra* plants and on plants infested with *Pieris brassicae* caterpillars. Time spent by an individual social wasp (mean + SD) on plots of *Brassica nigra* infested with late instar larvae of *P. brassicae* or on non-infested plots of plants (Wilcoxon matched pairs test, $\alpha = 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$; ns = non-significant) in late July (a) and late August (d) of 2012. Time spent by the social wasps processing prey (Mann Whitney U test, $\alpha = 0.05$; ** $P \leq 0.01$) (b). Successful attack rate by *P. dominula* and *Vespula sp.* relative to the total number of attacks to prey observed (Chi square test, $\alpha = 0.05$; ** $P \leq 0.01$) (c). N = number of individual insects observed.

VIDEO CLIP: Attack of a *P. brassicae* caterpillar by the social wasp *P. dominula*

http://youtu.be/2aNuw_RQ4ZY

Appendix E

Seed set of mustard plants is compromised in the absence of natural enemies of herbivores

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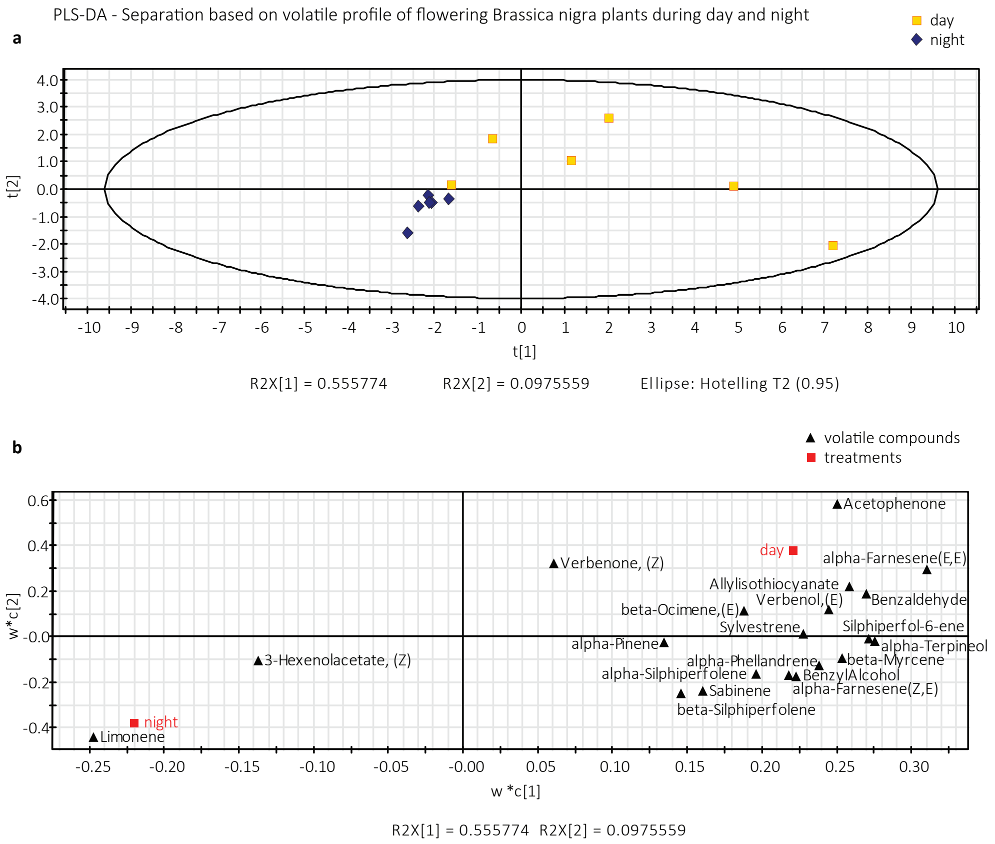
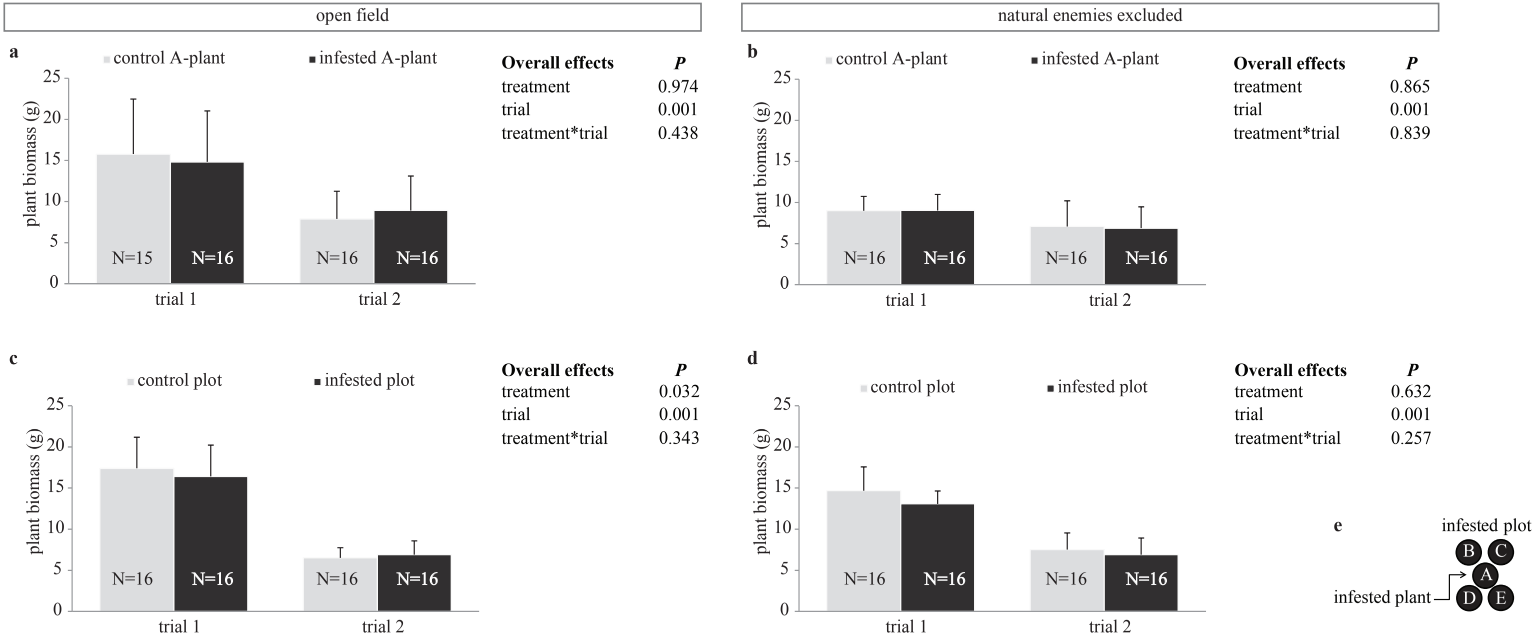


Fig. E1 Projection to Latent Structures Discriminant Analysis (PLS-DA) of volatile compounds collected from flowering *Brassica nigra* plants during daytime and night time. PLS-DA on the peak area of volatile compounds from headspace of *B. nigra* plants. a) Grouping pattern of samples according to the first two principal components, and the Hotelling's T^2 ellipse confining the confidence region (95%) of the score plot; b) Contribution of each of the volatile compounds to the first two principal components is shown in the loading plot of the PLS-DA components.

Table E1 Volatile compounds from flowering *Brassica nigra* plants collected during the day and night. *P* value at 0.05 level of significance is shown.

Volatile compounds	**Day** (peak area / plant biomass ± SD)*	**Night** (peak area / plant biomass ± SD)	***P value***
Benzenoids			
Acetophenone	8 ± 5	2 ± 1	0.087
Benzaldehyde	26 ± 17	-†	**0.014**
Benzyl alcohol	76 ± 73	4 ± 4	0.099
Monoterpenoids			
Limonene	-	8 ± 6	**0.046**
β-Myrcene	81 ± 53	3 ± 1	0.132
β-Ocimene, (*E*)-	100 ± 97	-	0.123
α-Pinene	71 ± 119	1 ± 1	0.818
α-Phellandrene	8 ± 3	-	0.083
Sabinene	34 ± 26	5 ± 1	**0.026**
Sylvestrene	72 ± 64	-	**0.047**
α-Terpineol	6 ± 3	-	**0.033**
Verbenol, (*E*)-	5 ± 1	ss‡	0.937
Verbenone, (*Z*)-	5 ± 3	2 ± 2	0.240
Sesquiterpenoids			
α-Farnesene, (*E*/*E*)-	10 ± 6	ss	**0.015**
α-Farnesene, (*Z*/*E*)-	6 ± 7	ss	0.074
7-*α*-H-Silphiperfol-5-ene	2 ± 2	ss	0.240
7-*β*-H-Silphiperfol-5-ene	8 ± 13	ss	0.180
Silphiperfol-6-ene	2 ± 3	ss	0.240
Fatty acid derivatives			
3-Hexen-1-ol, acetate, (*Z*)-	ss	6 ± 7	0.093
Sulfur containing compound			
Allyl isothiocyanate	15 ± 8	8 ± 7	**0.029**
Total volatile emission	**544 ± 505**	**44 ± 29**	**0.021**

*Values for peak area (mean ± SD) were divided by 10^5 and expressed per gram fresh weight. † (-) indicates that compound was not detected in any sample. ‡ (ss) indicates that the compound was detected in a single sample. Significant differences between means at the 0.05 level were determined using a Student's t-test or a Mann-Whitney U test, depending on data distribution, using square root transformed data. Differences in terms of total volatile emission were determined with a Wilcoxon signed-rank test, and the data were not transformed.



Overall effects	*P*
treatment	0.974
trial	0.001
treatment*trial	0.438

Overall effects	*P*
treatment	0.865
trial	0.001
treatment*trial	0.839

Overall effects	*P*
treatment	0.032
trial	0.001
treatment*trial	0.343

Overall effects	*P*
treatment	0.632
trial	0.001
treatment*trial	0.257

Fig. E2 Plant biomass (mean + SD) of *Pieris brassicae*-infested and non-infested control *Brassica nigra* plants in open field and when natural enemies of *P. brassicae* were excluded. In open field, plants and herbivores were exposed to naturally occurring pollinators and carnivorous insects. In the condition where natural enemies (carnivorous insects) were excluded, syrphid flies (*Episyrphus balteatus*) and male butterflies (*P. brassicae)* were used to ensure cross-pollination between plants of the same plot. Data are shown for the central plant (A-plant) of the plot (a,b) and at plot levels (c,d) for trials 1 and 2. Schematic representation of a field plot (e); each plot is composed of five plants, and the central plant of the plot (A-plant) was either infested with 30 *P. brassicae* eggs or was non-infested; second through fifth instar larvae colonised the other four plants of infested plots. A generalised linear model was used to determine significant differences at 0.05 levels. Number of plants and plots is shown in the bars.

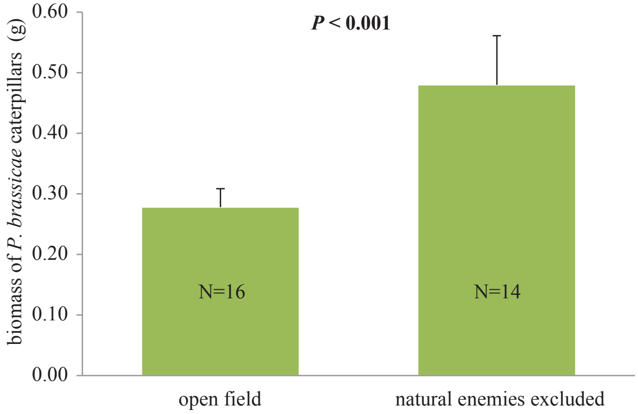


Fig. E3 Biomass (mean + SD) of fifth instar *Pieris brassicae* caterpillars reared on flowering *Brassica nigra* plants in open field conditions and in conditions where natural enemies of the herbivores were excluded. In open field conditions plants and herbivores were exposed to naturally occurring pollinators and carnivorous insects. In the condition where natural enemies (carnivorous insects) were excluded, plants of each plot were exposed to pollination by syrphid flies (*Episyrphus balteatus*) and male butterflies (*P. brassicae).* Number of plots of plants is shown in the bars - mean caterpillar weight of individuals recollected from a plot was the unit of replication. Caterpillars were recollected and weighed 28 days after egg deposition by the butterfly, for both the open field and exclusion conditions. Results are shown for trial 1 as no caterpillars were recovered for trial 2 in open field conditions.

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