Getting prepared for future attack

Induction of plant defences by herbivore *egg deposition* and consequences for the insect community

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To my family,
Στην οικογένειά μου,

Abstract

Plants have evolved intriguing defences against insect herbivores. Compared to constitutive defences that are always present, plants can respond with inducible defences when they are attacked. Insect herbivores can induce phenotypic changes in plants and consequently these changes may differentially affect subsequent attackers and their associated insect communities. Many studies consider herbivore-feeding damage as the first interaction between plants and insects.

The originality of this study was to start with the first phase of herbivore attack, egg deposition, to understand the consequences of plant responses to eggs on subsequently feeding caterpillars and their natural enemies. The main plant species used for most of the experiments was *Brassica nigra* (black mustard), which occurs naturally in The Netherlands. The main herbivore used was the lepidopteran *Pieris brassicae,* which lays eggs in clusters and feeds on plants belonging to the Brassicaceae family. This study investigated plant-mediated responses to oviposition and their effects on different developmental stages of the herbivore, such as larvae and pupae. Furthermore, the effects of oviposition were extended to four more plant species of the same family, and to higher trophic levels including parasitoids and hyperparasitoids. The experiments were conducted under laboratory, semi-field and field conditions. This study shows that *B. nigra* plants recognize the eggs of *P. brassicae* and initiate resistance against subsequent developmental stages of the herbivore. Interestingly, plant responses to oviposition were found to be species specific. Plants did not respond to egg deposition by another herbivore species, the generalist moth *Mamestra brassicae*. Moreover, most of the Brassicaceae species tested were found to respond to *P. brassicae* eggs, which indicates that plant responses against oviposition are more common among the family of Brassicaceae. To assess effects on other members of the food chain, the effects of oviposition on plant volatile emission and the attraction of parasitic wasps, such as the larval parasitoid *Cotesia glomerata*, were tested. It was shown that the wasps were able to use the blend of plant volatiles, altered by their hosts' oviposition, to locate young caterpillars just after hatching from eggs. The observed behaviour of the wasps was associated with higher parasitism success and higher fitness in young hosts. Similar results were obtained in a field experiment, where plants infested with eggs and caterpillars attracted more larval parasitoids and hyperparasitoids and eventually produced more seeds compared to plants infested with caterpillars only. This study shows that an annual weed like *B. nigra* uses egg deposition as reliable information for upcoming herbivory and responds accordingly with induced defences. Egg deposition could influence plant-associated community members at different levels in the food chain and benefit seed production. As the importance of oviposition on plant-herbivore interactions is only recently discovered, more research is needed to elucidate the mechanisms that underlie such plant responses and how these interactions affect the structure of insect communities in nature.

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General introduction and thesis outline

Foteini G. Pashalidou

Plant-insect interactions

The approximately 300,000 described plant species represent a large part of the biomass on our planet and form the trophic base of terrestrial food webs. Insects are estimated to represent around six million species (May 1988; Gaston 1991). About half of the insect species described to date, use plants as a food source (Schoonhoven *et al.* 2005). Plant-insect interactions, therefore, are among the most common interactions in terrestrial ecosystems and have evolved over more than 300 million years (Schoonhoven *et al.* 2005). Plant-insect interactions vary from mutualism to parasitism. Plants may also mediate interactions between different organisms, such as effects of aboveground insects on belowground organisms and *vice versa*. This can involve organisms at different trophic levels. Plants influence herbivorous insects and consequently modulate species diversity at higher trophic levels (Bukovinszky *et al.* 2008, Poelman *et al.* 2008). Natural enemies of the herbivorous insects, on the other hand, can change community dynamics via consumer-prey or parasitoid-host interactions and can consequently affect plant species diversity and abundance (Schmitz *et al.* 2004). Thus, bottom-up (plant-based) and top-down (natural enemies-based) selection forces both contribute to shaping insect populations (Gripenberg & Roslin 2007). In the following sections of the introduction, I describe: (a) general plant responses to feeding insects, (b) how plant responses to feeding insects can influence plant-associated insect communities, and (c) plant responses to egg deposition. Finally, I introduce the study system that was used to address the research objectives of this thesis and present the outline of the thesis.

Plant responses to insect herbivory

Even though plants cannot move, they are not passive when attacked by enemies. Plants have evolved a plethora of strategies to respond to herbivory. Plant traits that are **constitutively expressed** (i.e. morphological or phytochemical traits) are widely recognised to influence insect community composition (Dungey *et al.* 2000, Johnson *et al.* 2006, Whitham *et al.* 2006, Poelman *et al.* 2008). Constitutively expressed traits such as trichomes, thorns, and allelochemicals, however, might be costly for the plants in the absence of herbivores. Many plants respond upon herbivore attack with phenotypic changes (Adler & Karban 1994, Karban & Baldwin 1997, Agrawal & Karban 1999). Phenotypic changes due to plant-herbivore interactions are termed "**induced plant responses**" and they can be expressed locally at the site of herbivore attack or systemically throughout the plant (Agrawal & Karban 1999, Kessler & Baldwin 2002). Adler & Karban (1994) used a common framework where they compared three models of plant strategies against herbivory, i.e. constitutive defence, optimal inducible defence, and the "moving target." Plants with constitutive defences have a fixed defensive phenotype. Plants that respond with optimal inducible defence adopt a plastic phenotype where defence increases upon attack and decreases upon the termination of attack. In the moving target model, plants alter their phenotype in response to herbivory and these changes remain in the absence of an attack. Each of the three strategies is favoured under a certain set of environmental conditions and constraints. Constitutive defences are favoured under relatively constant herbivory, optimal inducible defences are favoured when herbivory rates and allocation costs are not too high. Last, the moving target strategy is favoured when allocations costs are not too high, herbivory rates are not constant, and plant phenotypes are effective against certain herbivores and not effective against others (Adler & Karban 1994).

Induced plant responses include changes in plant morphology, phytochemistry, and production of extrafloral nectar, e.g. in terms of plant chemistry, and consequently shape insect communities in time and space (Kessler & Halitschke 2007, Stam *et al.* 2014). Changes in nutritional quality (production of anti-digestive compounds or toxins) and morphology (hairs, cuticle etc.) directly affect herbivore preferences/performances. These changes are so-called **induced direct responses**.

Natural enemies (organisms at the third trophic level, e.g. parasitoids or predators) can use volatile organic compounds emitted by plants upon herbivory to locate their herbivorous hosts or prey, and might effectively reduce herbivore populations. Moreover, certain plant species produce extrafloral nectar upon herbivory that increases predator visitation and consequently may reduce herbivore numbers. These changes in plant phenotype that can indirectly affect herbivore performance are called **induced indirect responses**. Induced plant responses can be categorized into mechanisms that reduce herbivore preference/performance (**induced resistance**), and those that lead to a plant fitness benefit (**induced defences**). **Tolerance** is the ability of a plant to reduce the negative effects of herbivory (when it is approximately equally damaged compared to a susceptible host) and can repair, regrow or reproduce to a similar degree as undamaged conspecifics. An additional response of plants against herbivores is **phenological escape:** some plants (e.g. winter annuals) are not available to herbivores because they grow earlier in the season and escape herbivores that emerge later in the season.

Herbivore-induced changes in plant phenotype in a community context

Since more than two decades, it has been shown that different herbivore species can differentially induce changes in plant phenotypes and as a result, these altered phenotypes differentially affect subsequent herbivores and other community members (Kessler & Baldwin 2002, Kessler & Halitschke 2007, Bukovinszky *et al.* 2008, Poelman *et al.* 2008). Consequently, the expressed phenotype (e.g. changes in secondary metabolites, such as alkaloids, terpenoids, or glucosinolates) can influence the performance of the attacking herbivores and other organisms that colonize the plant (Agrawal 2000, De Vos *et al.* 2006, Poelman *et al.* 2008). As these plant-mediated interactions do not occur independently, it is fascinating how herbivores (receivers), which respond to plant induction can become inducers themselves and affect other herbivores or other community members, forming a complex network (Heidel-Fischer *et al.* 2014, Poelman & Dicke 2014). Such plant-herbivore interactions include spatially separated organisms (that feed e.g. below- or above-ground) and temporally separated organisms (some herbivores arrive earlier in the season than others) (Bezemer & van Dam 2005, Dicke *et al.* 2009).

Tobacco plants, for example, are able to redirect their defences when attacked by two different herbivores (Kessler & Baldwin 2004). When tobacco (*Nicotiana attenuata*) is attacked by myrid bugs (*Tupiocoris notatus*) or the tobacco hornworm (*Manduca sexta*) plants respond with changes at the transcriptomic level (i.e. transcriptional imprints). However, imprints of sequential or simultaneous attack were significantly different compared to single attack. Attack by both herbivores induced a change from growth-related to defence-related transcriptomic changes, whereas herbivore-specific changes occurred largely at primary metabolism and signalling cascades (Voelckel & Baldwin 2004). These exciting results from this study suggest the existence of a distinct defensive gene expression programme that produces different responses to a combination of biological stresses.

In Brussels sprouts (*Brassica oleracea* var. *gemmifera*) plants, aphids seem to facilitate the performance of subsequently attacking caterpillars (*Pieris brassicae*) while sharing the same host plant (Soler *et al.* 2012). Such effects extend further from above- to belowground interactions and *vice versa*. For example, when the fall armyworm *Spodoptera frugiperda* is the first colonizer of shoots of maize plants (*Zea mays*), belowground colonization by the western corn rootworm (*Diabrotica virgifera*) is constrained (Erb *et al.* 2011). Phenotypic changes in plant chemistry can also influence higher trophic levels depending on the sequence of the arrival of upcoming colonizers. For example, when non-host herbivores share the same plant with herbivorous hosts of parasitoids, changes in the induced volatile blends might interfere with parasitoid host-location behaviour (Dicke *et al.* 2009, de Rijk *et al.* 2013). Our knowledge is limited when induced changes in plant phenotypes are extended beyond sequences of more than two herbivores. However, we can use the knowledge gained from pairwise interactions to test whether plants are limited in their responses regarding the number of herbivores or in the sequence of arrival of these herbivores. There are two main hypotheses: (a) plants are limited in their responses after the first attacker and canalize their phenotype, regardless of the subsequent attacker (Viswanathan *et al.* 2005) or (b) plants might be able to redirect their defences towards the new attacker (Poelman & Dicke 2014).

Plant responses to egg deposition

Many studies of induced plant responses to herbivory considered feeding damage as a first interaction between the insect and the plant. However, egg deposition often precedes feeding damage in many herbivores, i.e. in the majority of lepidopteran and sawfly species. To date, plant responses to egg deposition have been described for more than 20 insect species of the five major insect orders in more than 20 different plant species ranging from mono- to dicotyledonous species and from gymnosperms to angiosperms (Hilker & Fatouros 2015). In most of the described studies, plants kill herbivore eggs, either directly by, e.g. chemical or physical defences, or indirectly by attracting parasitoids that kill the insect embryo. Only in some cases, eggs have been shown to act as a signal for the plant indicating upcoming herbivory. Interestingly, herbivores can adapt and manipulate plant responses and suppress them to the herbivore's own benefit. These different types of induced responses to egg deposition are described in detail below.

Direct and indirect plant responses to egg deposition

What is fascinating regarding plant responses to herbivorous insect eggs is that these defence responses are mostly detrimental for the eggs. Unlike feeding herbivores that can move away from the plant that induces the production of toxins, eggs cannot move. Therefore, plant responses to eggs can actually kill them and prevent feeding damage caused by the hatching larvae. Several strategies of how plants can kill the eggs of their enemies have been reported. For example, a hypersensitive response (HR)-like necrosis in black mustard (*Brassica nigra*) plants to eggs of its attackers, Cabbage White butterflies (*Pieris* spp.) (Shapiro & DeVay 1987, Fatouros *et al.* 2012). Hypersensitive response or programmed cell death has originally been described as a plant response to microbial pathogens, and it is usually expressed by rapid cell death that results in necrotic lesions in and around the infection site (Lam *et al.* 2001). *Brassica nigra* responds to the eggs of its hosts with a similar response: necrotic lesions surround the eggs of the butterflies and some of these eggs shrink and desiccate and/or drop off the plant (Fig. 1a). The mechanism that underlies HR-like necrosis against herbivore eggs is still largely unknown.

A study by Little *et al.* (2007) has shown that *Arabidopsis* plants respond strongly with callose accumulation and production of H_2O_2 at the *Pieris* oviposition site. Callose has been shown to act as a seal in wound sites in the plant and H_2O_2 plays a role in HR induction (Levine *et al.* 1994). Moreover, *Pieris* oviposition-induced gene expression changes in *Arabidopsis* were similar to gene expression changes during bacteria-induced HR (Little et al. 2007, Bruessow and Reymond 2007). Other example of egg-killing responses are described in particular lines of *Pisum sativum*, which form neoplasms in response to eggs of *Bruchus pisorum* or *Callosobruchus maculatus* and subsequently the eggs drop from the plant (Doss *et al.* 1995). Similarly, *Physalis angulata* forms neoplasms underneath the eggs of *Heliothis subflexa* (Petzold-Maxwell *et al.* 2011) (Fig. 1b). Another strategy is the production of wound tissue that leads to egg crushing: the leaf beetle *Pyrrhalta viburnum* lays its eggs in little cavities in *Viburnum* twigs and covers them with faeces and plant material to protect them against predators (e.g. ants). However, when the twigs of the plant respond with wound tissue to the oviposition, the eggs are squeezed or squashed inside the cavity (Desurmont *et al.* 2011) (Fig. 1c). Similar wound tissue responses are known for other systems such as the jarrah leafminer moth *Perthida glyphopa* that lays its eggs on jarrah leaves (*Eucalyptus marginata*) or *Anastrepha* fruit flies that lay their eggs on fruits of cultivated avocado (*Persea americana*) (Aluja *et al.* 2004). Finally, rice plants produce ovicidal substances that can kill eggs of herbivorous insects. Some *japonica* rice varieties (*Oryza sativa* var. *Reiho*) respond to eggs of particular planthopper species such as *Sogatella furcifera*, *Nilaparvata lugens*, and *Laodelphax striatellus* by forming watery lesions (Seino *et al.* 1996, Suzuki *et al.* 1996). The study by Suzuki *et al.* (1996) on rice and *S. furcifera* planthoppers showed that rice plants produce an ovicidal compound, benzyl benzoate that reduces the survival of *S. furcifera* eggs from about 90 to only 20 %.

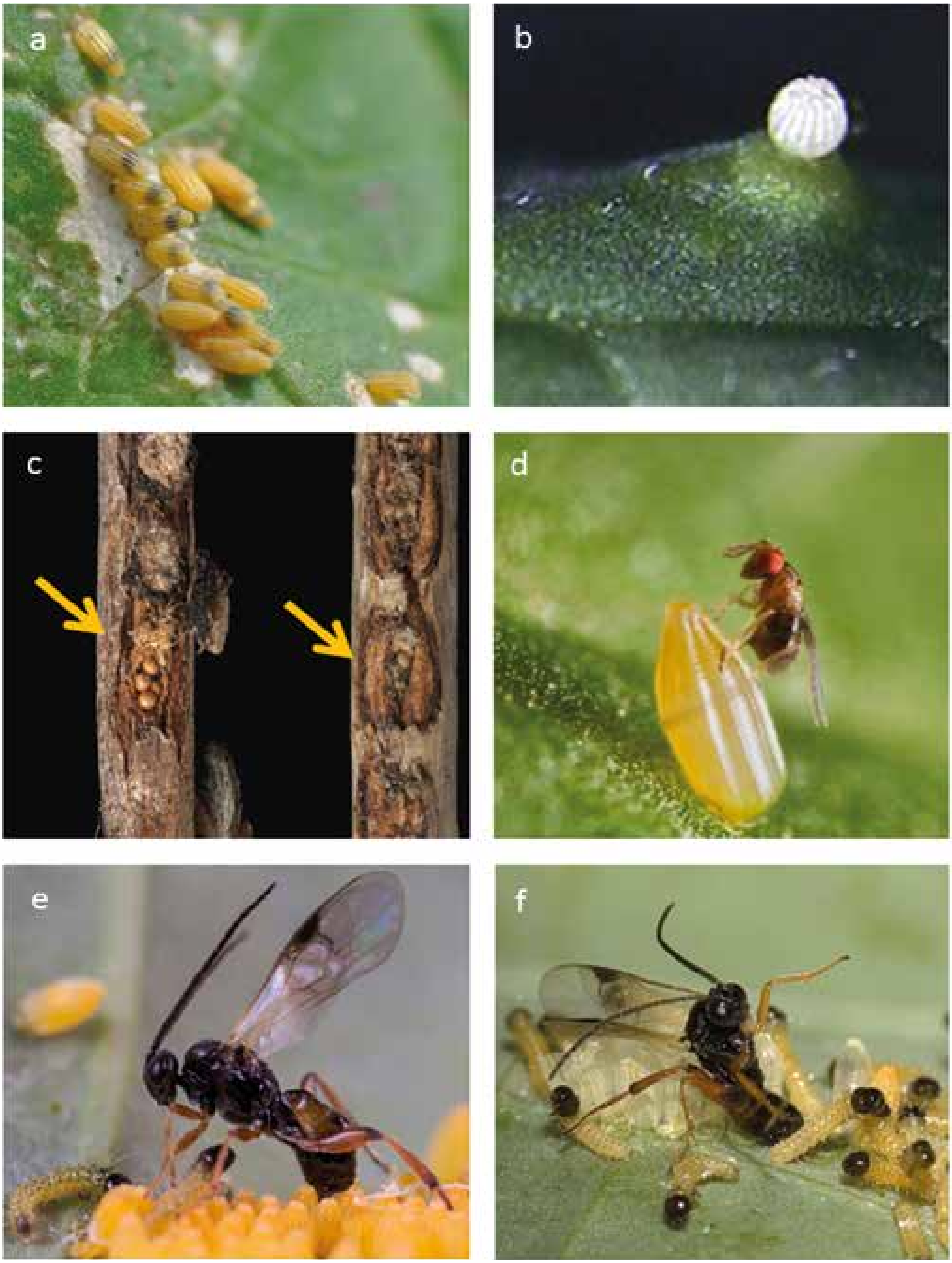

Figure 1. Plant responses induced by herbivorous insect eggs. (a) Eggs of the Large Cabbage White butterfly (*Pieris brassicae*) surrounded by necrotic tissue (hypersensitive-like response) in *Brassica nigra* plants. (b) Eggs of *Heliothis subflexa* and neoplasmatic cell formation in *Physalis angulata* plants. (c) Eggs of Viburnum leaf beetle, *Pyrrhalta viburni*, which are laid in a cavity inside a twig of a *Viburnum* plant, on the right side the wound tissue that crushes the eggs is visible. (d) *Trichogramma* egg parasitoids attracted by oviposition induced plant volatiles. Larval parasitoid *Cotesia glomerata* parasitising neonates of *P. brassicae* during hatching (e) or just after hatching (f) from the eggs (Figure modified from Hilker & Fatouros 2015).

Almost two decades ago, the first studies demonstrated that plants respond to deposition of eggs by herbivorous insects with the production of volatiles attracting enemies of their own enemies (Meiners & Hilker 1997, Meiners & Hilker 2000). Enemies of the enemies are egg parasitoids, parasitic wasps that eventually kill the embryo inside the eggs (Fig. 1d).

These first studies were conducted on elm (*Ulmus minor)* and later on pine (*Pinus sylvestris*). In both cases, it was shown that specialist egg parasitoids, *Oomyzus gallerucae* and *Closterocerus ruforum*, which attack the elm leaf beetle *Xanthogaleruca luteola* and the pine sawfly *Diprion pini* respectively, use oviposition-induced plant volatiles (OIPVs) to locate their hosts (Meiners & Hilker 1997, Meiners & Hilker 2000, Hilker *et al.* 2002, Mumm *et al.* 2003, Mumm *et al.* 2005). More studies have revealed that egg parasitoids of lepidopteran species make use of OIPVs, such as *Trichogramma* spp. locating eggs of specialist butterflies on black mustard plants (*Brassica nigra*) (Fatouros *et al.* 2012). *Trichogramma* wasps also use OIPVs to locate the eggs of a moth (*Chilo partellus*) on maize landraces (Tamiru *et al.* 2011)(Fig. 1d). Unlike leafhoppers, hemipteran bugs, sawflies, and beetles, lepidopterans (butterflies and moths) usually do not damage the plant tissue before or during oviposition (Hilker & Fatouros 2015). Moreover, egg parasitoids can be arrested by close-range plant cues induced by egg deposition: e.g. eggs of *Pieris* spp. change the leaf surface chemistry in *Brassica oleracea* var. *gemmifera* or *Arabidopsis* resulting in *Trichogramma* parasitoids searching longer in the egg-infested leaf area (Fatouros *et al.* 2005, Pashalidou *et al.* 2010, Blenn *et al*. 2012). As a result, egg parasitoids have a higher probability of encountering a possible host.

Egg deposition acts as a signal of upcoming herbivory

Just like animals, plants can also sense chemical cues (Karban 2008). Volatiles from neighbouring plants damaged by herbivores induce defences (Conrath *et al.* 2006). Even in the same plant, undamaged parts respond to cues from damaged parts, a phenomenon called systemic response (Conrath *et al.* 2006, Heil & Ton 2008). Plants can use any reliable cue that indicates future attack by pathogens or insects to strengthen or accelerate its response to attackers. The induction of this physiological state is called “priming” (Conrath *et al.* 2006, Frost *et al.* 2008). This adaptation of plants to respond with a primed state to cues that reliably indicate future herbivory, might give plants an evolutionary advantage in the potential arms race with their herbivores (Conrath *et al.* 2006). Eggs might serve as reliable cues for plants, since eggs indicate that feeding damage will start as soon as the larvae hatch. Therefore, it is expected that eggs are reliable predictors of upcoming herbivory (Hilker & Fatouros 2015). Plants have evolved responses that target exclusively the eggs (see previous section: “*Direct and indirect plant responses to egg deposition*”).

Recently, however, plants have been also shown to recognize the eggs of specific herbivores and respond stronger and more effectively against the feeding larvae (Beyaert *et al.* 2011, Geiselhardt *et al.* 2013).

More effectively means here that either the larvae show higher mortality, or lower performance on plants previously exposed to eggs compared to larvae feeding on egg-free plants. Higher efficiency of such plant-mediated effects of egg deposition against forthcoming herbivory has been shown in the *Arabidopsis thaliana - P. brassicae* system and in the *Pinus sylvestris – Diprion pini* system (Beyaert *et al.* 2011, Geiselhardt *et al.* 2013). In addition, egg deposition results in early attraction of parasitoids that do not kill the eggs but parasitise the larvae inside the eggs or upon emerging from the eggs (Fig. 1e, f). Being on time seems an important factor in host location of these egg-larval or larval parasitoids, and in interactions with the host plant. As plant-volatile emission, changes with time (Turlings *et al.* 1998, Clavijo McCormick *et al.* 2012), parasitoids are expected to be able to use them to locate high-quality hosts. In addition to the responses described so far, recent studies show that plants can also use egg deposition as a reliable cue to accelerate reproduction and overcome herbivory. In more detail, flowering *B. nigra* plants have been shown to escape and safeguard their offspring from their voracious enemies (i.e. feeding *P. brassicae* caterpillars) in a remarkable way. Plants accelerate their reproduction with faster and increased seed production before the caterpillars start to consume the reproductive organs of the plant, i.e. the flowers (Lucas-Barbosa *et al.* 2013). Thus, plants might anticipate upcoming herbivory by using herbivorous eggs as a predictive factor for feeding damage and influence (a) direct plant defences against the feeding larvae, (b) attraction of parasitoids and (c) reproductive escape.

Egg deposition can suppress plant responses to subsequent herbivory

In contrast to the studies explained in the previous paragraph, describing how plants respond to eggs, there are studies that show contradictory results. These studies indicate that herbivores may counter-adapt to these responses e.g. by suppressing them or by aggregating eggs at the oviposition site (Bruessow *et al.* 2010, Desurmont & Weston 2011, Desurmont *et al.* 2014).

Interestingly, when *A. thaliana* was treated with an egg extract of *P. brassicae*, feeding larvae of the generalist *Spodoptera littoralis* gain more weight compared to feeding larvae on untreated plants. However, the performance of *P. brassicae* remained similar on treated and untreated plants (Bruessow *et al.* 2010). This study showed that egg deposition can suppress plant defences against feeding larvae of a generalist, however, the specialist remained unaffected to this egg-mediated plant response (Bruessow *et al.* 2010). When a commercial maize variety (cv. Delprim) was treated with *S. frugiperda* eggs followed by additional artificial wounding, the plants produced a lower amount of volatiles compared to egg-free plants (Peñaflor *et al.* 2011). However, lower emission of volatiles does not necessarily mean suppression of indirect defence. Some studies have shown that even though plant volatiles are suppressed due to oviposition, natural enemies can still successfully use them to locate and parasitise their hosts (Bruce *et al.* 2009, Fatouros *et al.* 2012, Tamiru *et al.* 2012).

Study system

This thesis focussed on plants of the Brassicaceae family. This family contains many important agricultural crops like cabbage, cauliflower, mustards, broccoli, as well as the model plant *Arabidopsis thaliana*. Brassicaceous plants are known to produce secondary plant metabolites that are involved in direct and indirect plant responses (Bukovinszky *et al.* 2005, Shiojiri *et al.* 2006, Hopkins *et al.* 2009, Mumm & Dicke 2010). Their major secondary metabolites are glucosinolates (GS), which result in toxic products upon tissue damage. These toxins, isothiocyanates and nitriles, can significantly alter the physiology and development of herbivores (Hopkins *et al.* 2009). Brassicaceous plants exhibit a large variation in direct and indirect responsive traits against herbivory. Therefore, they represent an ideal system to address the fundamental objectives of this thesis. The Laboratory of Entomology has a long history studying tritrophic interactions in the Brassicaceae-*Pieris* system (see PhD theses of, e.g.: Geervliet (1997), van Poecke (2002), Broekgaarden (2008), Bruinsma (2008), Gols (2008), Poelman (2008), Snoeren (2009), Kos (2012)). Gols (2008) showed that specialist herbivores and their parasitoids are affected less severely by high GS concentrations, compared with generalist herbivores and their parasitoids.

Furthermore, Broekgaarden (2008) showed that there is intraspecific variation in the performance of two different herbivores (i.e. *Pieris rapae* caterpillars and *Brevicoryne brassicae* aphids) within *Brassica oleracea* cultivars. In addition, she showed interspecific variation in performance of the same herbivores, which were linked to transcriptional differences with respect to feeding, when comparing *B. nigra* with *B. oleracea*. The differences in transcriptional responses induced by the two herbivores after feeding may result from the differences in feeding modes of these insects. *Pieris rapae* feeding caused activation of genes affected by the plant hormone jasmonic acid (JA) (e.g. *LOX2*), whereas *LOX2* expression was not induced after *B. brassicae* feeding. Bruinsma (2008) studied the role of jasmonic acid in herbivore-induced responses. Jasmonic acid is a key plant hormone, involved in plant responses to insect herbivores. Application of JA triggers plant responses that are similar but not identical to plant responses to herbivores. Bruinsma (2008) found that plants exposed to feeding by herbivores (*Pieris spp.*) and plants treated with JA were different in their chemistry. Finally, *Brassica oleracea* cultivars differ in resistance against specific herbivores and the susceptible cultivars harboured a higher herbivore diversity compared to the resistant ones in the field (Poelman, 2008).

In chapters 2, 3, 4, and 5, the wild crucifer *B. nigra* L. (Fig. 2) was selected for laboratory, semi-field, and field experiments. *Brassica nigra* (or black mustard) is a wild Eurasian annual that often carries trichomes and naturally occurs in The Netherlands. It usually occurs from May until October and it is considered an obligatorily outcrossing species (Conner & Neumeier 1995). *Brassica nigra* occurs along river valleys and coastal habitats (Bischoff & Tremulot 2011). In chapter 3, four additional wild species of Brassicaceae were used, all known to interact with the Large Cabbage White butterfly, *P. brassicae* L. (Lepidoptera: Pieridae) (Fig. 2) (Courtney & Chew 1987). *Pieris brassicae,* a specialist on plants of the Brassicaceae family, was used as the main herbivore species (Feltwell 1982, Courtney & Chew 1987). It is a gregarious species native to The Netherlands. In chapter 2, I also studied *Mamestra brassicae* L. (Lepidoptera: Noctuidae) (Fig. 2), which is a generalist gregarious moth, that feeds upon many brassicaceous species (Harvey & Gols 2011). Finally, in chapter 5, I used the gregarious larval endoparasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae) (Fig. 1e, f, Fig. 2), which is specialized on young caterpillars of Cabbage White butterflies.

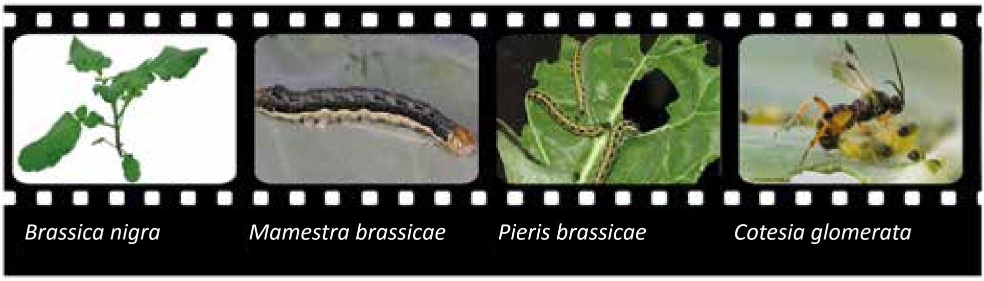

Figure 2. The tritrophic system used in the present thesis. *Brassica nigra* plants were used in the vegetative stage. In chapter 2, the performance of *M. brassicae* and *P. brassicae* herbivores was followed up to the second larval instar (here presented as last instar and neonates respectively). In chapter 3, *P. brassicae* was followed up to pupation after feeding on different wild Brassicaceae species. In chapter 4, *C. glomerata* wasp behaviour was tested in response to egg-plant interactions (here *C. glomerata* is presented while parasitising just hatched neonates of *P. brassicae*).

Research objectives

As described above, plants have evolved highly sophisticated systems to recognize specific attackers and respond accordingly. Nonetheless, many studies that have investigated plant responses to lepidopteran insects, introduce the herbivores as larvae on the plants without exposing the plants to the initial phase of herbivory (i.e. oviposition). Moreover, many previous studies have focused on a single attacker and its parasitoids and plant responses were investigated at one specific time point since the initial attack. Under natural conditions, however, plants are exposed to multiple stressors (herbivores at different developmental stages, different feeding guilds etc.) and subsequently exhibit plasticity in their responses over time in order to successfully defend themselves (Stam *et al.* 2014).

In this thesis, I investigated whether egg deposition by one of two lepidopteran species, can modify later plant responses to feeding caterpillars (Fig. 3). This insect-plant combination system (Fig. 3) offers an ideal opportunity to test the effect of insect egg deposition on subsequent insect attack in a plant-associated insect community context. This study aimed to understand the temporal dynamics of plant defences induced by herbivorous insects. In addition, I studied how plant-mediated effects alter insect communities using a multidisciplinary approach that involves behavioural, chemical, and field studies.

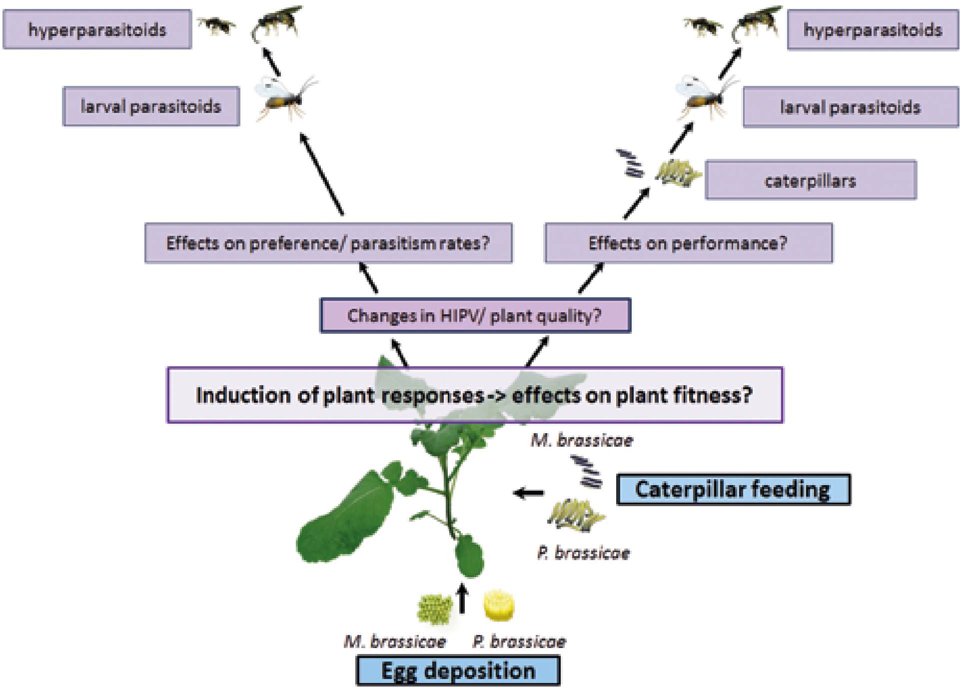

Figure 3. Main objectives addressed in this thesis. In *Brassica nigra*, egg deposition by a specialist butterfly may induce plant responses that either alter plant quality and the performance of subsequently feeding herbivores and their parasitoids and hyperparasitoids, or induce specific volatiles i.e. herbivore-induced plant volatiles, which affect the preferences / parasitism rates of larval parasitoids and hyperparasitoids, and ultimately affect plant fitness.

The **main objective** of my thesis was to investigate whether egg deposition by a butterfly induced plant responses that affected subsequently feeding larvae and their parasitoids in the wild crucifer *Brassica nigra*, at different levels of complexity from bitrophic interactions to plant-associated insect communities. In addition, I increased the number of inducing herbivores by including a generalist moth, and the number of plant species by including four more brassicaceous species, to test how specific and persistent oviposition-induced plant responses are. I **hypothesised** that egg deposition by specialist lepidopteran species induces phenotypic changes in *B. nigra* plants that affect subsequent herbivore attackers and their natural enemies.

Thesis outline

This thesis addressed the effects of egg deposition on plant responses to subsequent herbivory in a community context.

In **chapter 2**, laboratory and semi-field experiments were presented on a direct plant response of *B. nigra* plants against two different lepidopteran species, i.e. a butterfly (*Pieris brassicae*), and a moth (*Mamestra brassicae*), when plants had been exposed to previous egg deposition. In particular, I investigated whether plants exhibit a specific response to the initial attacker (specificity of induction) and whether this response differentially affected the subsequent attackers (specificity of effect). I assessed the performance in terms of growth of conspecific and heterospecific caterpillars at different time points since the start of feeding, either on plants previously exposed to eggs (eggs(+)) or on egg-free plants (eggs(-)). I also assessed plant development from the moment of egg deposition until caterpillars had reached the end of the second larval stage by measuring plant height and flowering time. Based on the results described in **chapter 2**, in **chapter 3** I expanded the number of wild Brassicaceae species studied, in order to test how common and persistent these plant-mediated effects of oviposition by *P. brassicae* are. The following brassicaceous plant species were investigated in this chapter: *B. nigra*, *Sinapis arvensis, B. oleracea, Moricandia moricandioides,* and *M. arvensis.* I assessed the performance of the herbivore based on herbivore biomass in two life stages (larvae and pupae) and development time from egg to adult.

In **chapter 4** was addressed the temporal dynamics of plant volatile emission of *B. nigra* induced by oviposition (OIPVs) and by larval feeding (HIPVs) of *P. brassicae*. Moreover, I investigated how these changes in plant volatiles over time were linked with the preference and performance of a larval parasitoid under controlled laboratory conditions. In two-choice bioassays, the attractiveness of the two different treatments (eggs(+) and eggs(-) plants) to a parasitoid wasp (*Cotesia glomerata*) was tested at different time points (just before larval hatching and up to three days after hatching). The headspace of plants exposed to the two different treatments was collected and HIPV-emission was compared for the same time points at which the behavioural responses of parasitoids were assessed.

Furthermore, the fitness of the parasitoid was linked to odour preferences by assessing biomass and parasitism success as proxies. In the previous chapters, I identified the plant-mediated effects of *P. brassicae* egg deposition on preference and/or performance of herbivores and their primary parasitoids in the laboratory.

Subsequently, in **chapter 5,** I studied how plant responses to herbivore egg deposition or a chemical elicitor affect plant-associated insect communities and plant fitness in the field. This chapter was tested whether previous *P. brassicae* egg deposition or application of the chemical elicitor benzyl cyanide changed plant quality and/or volatile emission and subsequently affects (a) the performance of the caterpillars and their parasitoids and hyperparasitoids, (b) the parasitism rates and community structure of parasitoids and hyperparasitoids, and (c) plant fitness.

Finally, in **chapter 6** the findings of this thesis were discussed, with an emphasis on integration of the results from the laboratory to the field. Here, I discussed how plant responses to oviposition of herbivorous insects affect the plant phenotype and how changes in plant phenotype affect plant-associated insect communities. I focused on the role of plant responses to oviposition by specialist or abundant herbivores, which seems to be underestimated in insect-plant interactions so far. Finally, I presented an outline for future directions in unravelling the possible mechanisms that underlie egg-induced plant responses. In addition, I discussed the value of information presented in this thesis for assessing ecological effects that underlie plant-associated insect communities with the use of a multidisciplinary approach.

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Chapter 2

Phenotypic plasticity of plant response to herbivore eggs: effects on resistance to caterpillars and plant development

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Herbivory induces direct resistance responses in plants that negatively affect subsequently colonizing herbivores. Moreover, eggs of herbivorous insects can also activate plant resistance, which in some cases prevents hatching larvae from feeding. Until now, plant mediated effects of eggs on subsequent herbivory, and the specificity of such responses, have remained poorly understood. We studied the specificity and effects of plant resistance induced by herbivore egg deposition against lepidopteran larvae of species with different dietary breadths, feeding on a wild annual plant, the crucifer *Brassica nigra*. We examined whether this plant-mediated response affects the growth of caterpillars of a specialist (*Pieris brassicae*) that feeds on *B. nigra* leaves and flowers, and a generalist (*Mamestra brassicae*) that rarely attacks this wild crucifer. We measured growth rates of neonate larvae to the end of their second instar after the larvae had hatched on plants exposed to eggs vs. plants without eggs, under laboratory and semi-field conditions. Moreover, we studied the effects of egg deposition by the two-herbivore species on plant height and flowering rate before and after larval hatching. Larvae of both herbivore species that developed on plants previously infested with eggs of the specialist butterfly *P. brassicae* gained less mass compared with larvae that developed on egg-free plants. Plants exposed to butterfly eggs showed accelerated plant growth and flowering compared to egg-free plants. Egg deposition by the generalist moth *M. brassicae*, in contrast, had no effect on subsequent performance by either herbivore species, or on plant development. Our results demonstrate that B. nigra plants respond differently to eggs of two herbivore species in terms of plant development and induced resistance to caterpillar attack. For this annual crucifer, the retardation of caterpillar growth in response to deposition of eggs by *P. brassicae* in combination with enhanced growth and flowering likely result in reproductive assurance, after being exposed to eggs from an herbivore whose larvae rapidly reduce the plant's reproductive potential through florivory.

Keywords*:* *Brassica nigra*, direct defence, hypersensitive response, *Mamestra brassicae*, oviposition induced plant defence, phenotypic plasticity, *Pieris brassicae*, priming.

Introduction

Plants have evolved distinct defence mechanisms against biotic stresses. They respond to herbivore damage with induced resistance, which affects subsequent attackers and their natural enemies. This phenomenon is widespread across the plant kingdom (Karban & Baldwin 1997). Induced direct resistance traits negatively affect the herbivore's behaviour or performance (Kessler & Baldwin 2002, Chen 2008). Indirect resistance traits include the attraction of natural enemies, such as parasitoids and predators that kill the attacking herbivores, e.g. by herbivore-induced plant volatiles (HIPVs) (Dicke & Baldwin 2010, Kessler & Heil 2011). In order to understand the ecology and evolution of induced resistance in plants, the specificity of the response can be categorized in two types (Karban & Baldwin 1997): (a) specificity of induction or elicitation meaning that plants exhibit a specific response to the initially attacking herbivore and (b) specificity of effects meaning that an induced phenotype has different consequences for different herbivores in terms of performance (Stout *et al.* 1998, Agrawal 2000, van Zandt & Agrawal 2004).

The vast majority of documented cases on induced resistance to herbivory concerned a response to feeding damage. Recently, an increasing number of studies have demonstrated induced plant responses to egg deposition, which is the initial phase of plant colonization for many herbivorous insects (Beyaert *et al.* 2011, Desurmont & Weston 2011, Peñaflor *et al.* 2011, Petzold-Maxwell *et al.* 2011, Tamiru *et al.* 2011, Blenn *et al.* 2012, Fatouros *et al.* 2012, Lucas-Barbosa *et al.* 2012). Insect eggs represent a future threat for plants. From an ecological point of view, it is highly important for the plants to respond "in time" to egg deposition because it can prevent herbivores from feeding and developing (Hilker & Meiners 2011). Plants can directly respond to egg deposition by neoplasm formation underneath the eggs (Doss *et al.* 1995, 2000, Petzold-Maxwell *et al.* 2011), hypersensitive response (HR) previously reported as an active defence of plants against biotrophic pathogens (Shapiro & DeVay 1987, Balbyshev & Lorenzen 1997, Pontier *et al.* 1998, Hilker & Meiners 2006, Petzold-Maxwell *et al.* 2011), induction of ovicidal substances (Seino *et al.* 1996) or induction of wound tissue that leads to egg crushing (Desurmont & Weston 2011). Oviposition can also induce indirect resistance such as the emission of plant cues that recruit egg parasitoids that parasitise the embryo and consequently kill it (Meiners & Hilker 2000, Hilker *et al.* 2002, Colazza 2004, Fatouros *et al.* 2008b, Fatouros *et al.* 2009, Pashalidou *et al.* 2010, Tamiru *et al.* 2011, Blenn *et al.* 2012). In the present study, we determine the specificity of induced plant resistance in response to oviposition by attackers of different dietary breadth. Specialist and generalist herbivores might respond differently towards induced plant resistance (Karban &

Baldwin 1997, Ali & Agrawal 2012). Constitutive plant resistance is usually more effective against generalists than against specialists, because specialists have co-evolved with a specific host plant (Ehrlich & Raven 1964).

Here, we investigate whether *Brassica nigra* plants respond to egg deposition by two different herbivores (specificity of induction) and whether induced resistance affects subsequently feeding larvae of both herbivores (specificity of effect). In a laboratory and semi-field experiment we used the black mustard plant, *B. nigra*, the generalist cabbage moth, *Mamestra brassicae* (Lepidoptera: Noctuidae) and the specialist Large Cabbage White butterfly, *Pieris brassicae* (Lepidoptera: Pieridae) (Fig. 1). *Brassica nigra* is a wild Eurasian summer annual crucifer, native to The Netherlands; it occurs from May until October and it is considered an obligatorily out-crossing species (Conner & Neumeier 1995). *Brassica nigra* plants usually occur along field edges, in river valleys and coastal habitats (Bischoff & Tremulot 2011).

As a member of the Brassicaceae, *B. nigra* produces glucosinolates, defensive compounds that reduce growth and survival of generalist herbivores (Gols & Harvey 2008, Hopkins *et al.* 2009). Upon tissue damage, the enzyme myrosinase hydrolyses glucosinolates and converts them to toxic isothiocyanates (Rask *et al.* 2000, Wittstock *et al.* 2004). *Mamestra brassicae* is a generalist herbivore feeding on cabbages and other crops (Harvey & Gols 2011a). *Pieris brassicae* feeds upon many species within the Brassicaceae, including *B. nigra* (Harvey *et al.* 2010). Both herbivores are gregarious species, native to The Netherlands. Previous studies have shown that *P. brassicae* oviposition can change the expression of hundreds of genes related to plant defence and plant stress in the crucifer *Arabidopsis thaliana* (Little *et al.* 2007). Furthermore, *Pieris* egg deposition also induces transcriptomic changes in *B. oleracea* plants that may explain the arrestment of *Trichogramma* egg parasitoids to egg-induced leaves (Fatouros *et al.* 2008a).

So far, however, little is known on how oviposition affects the performance of feeding larvae that hatch from the eggs (Bruessow *et al.* 2010, Beyaert *et al.* 2011). Our first objective was to study whether oviposition by two herbivore species induces resistance in *B. nigra* against subsequently feeding larvae. We assessed the weight of conspecific and heterospecific caterpillars at different time points since the start of feeding, either on plants previously exposed to eggs or on egg-free plants. Our second objective was to evaluate the effects of oviposition on plant growth and flowering until caterpillars reached the end of the second larval stage. Furthermore, we assessed (a) plant growth from egg hatching until the moment caterpillars had reached the end of the second larval stage and (b) the proportion of plants that passed from the vegetative to the reproductive phase i.e. plants with open flowers, just before the eggs hatched.

Materials & Methods

Plants and insects

Black mustard plants (*Brassica nigra* L.) were grown in a greenhouse (23 ± 2°C, 70% r.h., L16:D8). Seeds were obtained from the Centre of Genetic Resources, Wageningen (accession number: CGN06619), The Netherlands, and had been multiplied by exposing them to pollinators in the surroundings of Wageningen. Four-week-old non-flowering plants were used for the experiments. The Large Cabbage White butterfly, *Pieris brassicae* L. (Lepidoptera: Pieridae) and the cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae; see Plate 1) were reared on *B. oleracea* var. *gemmifera* L. cv. Cyrus plants in a climatised room (21±1°C, 50–70% relative humidity, L16:D8).

Plant treatments

Plants were infested either with *P. brassicae* or *M. brassicae* eggs ("eggs(+) plant", i.e. plant exposed to egg deposition). Each eggs(+) plant was placed in a cage with approximately 50 female *P. brassicae* butterflies. Oviposition was observed usually for 10 min until approximately 20 eggs were laid on the fourth or fifth leaf.

Then, the plant was removed from the cage and eggs in excess of 20 (if any) were removed through gently rubbing with a fine brush. We observed the oviposition behaviour of butterflies in order to minimize differences in initial numbers of eggs among eggs(+) plants.

Because *M. brassicae* is a nocturnally active moth, direct observation of oviposition was not feasible. A plant was placed in a cage with 4-5 *M. brassicae* overnight, to allow oviposition. The following morning 20 eggs were kept per eggs(+) plant laid on the fourth or fifth leaf. Plants that had not been in contact with butterflies or any other insect were used as a control ("eggs(-) plant", i.e. a plant not exposed to egg deposition). All plants were kept in a greenhouse compartment (23 ± 2°C, 70% relative humidity, L16:D8) in cages for 3 days and then transferred to a climate chamber (21 ± 2ºC, L16:D8, 83 µmol m^{-2} s^{-1} PAR, photosynthetically active radiation). All plants infested with *P. brassicae* eggs were checked for hypersensitive response (HR), i.e., necrotic tissue around the eggs. Eggs(+) plants expressing HR were marked as HR+ and plants that did not express HR were marked as HR-. This discrimination was made in order to clarify if HR had an effect on plant responses against feeding caterpillars. None of the *B. nigra* plants infested with *M. brassicae* eggs expressed HR (see also Fatouros *et al.* 2012).

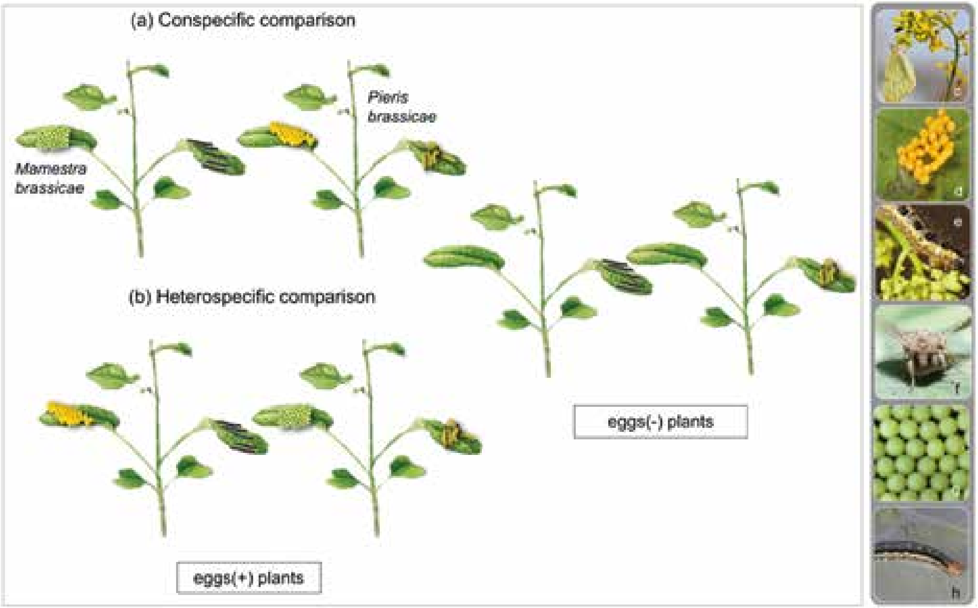

Figure 1. Plant treatments and insects used in the experiments. (a) *Pieris brassicae* or *Mamestra brassicae* caterpillar body mass were measured and compared between caterpillars feeding on plants previously infested with eggs (i.e. eggs(+)) of the same species and caterpillars feeding on plants that had not been exposed to egg deposition (i.e. eggs(-). (b) *P. brassicae* or *M. brassicae* caterpillar weight was measured and compared between caterpillars feeding on plants previously infested with eggs of the other species and caterpillars feeding on plants. Yellow eggs: *P. brassicae*, green eggs: *M. brassicae;* (*Brassica nigra* illustration: Alison Schroeder). Different herbivorous insect species used for this study: (c) female *P. brassicae* on flowers of *B. nigra*, (d) *P. brassicae* egg clutch on *B. nigra* with surrounding necrosis due to a hypersensitive response (HR), (e) fifth instar *P. brassicae* caterpillar feeding on flower buds of *B. nigra*, (f) adult *M. brassicae* moth, (g) egg clutch of *M. brassicae*, and (h) fifth instar *M. brassicae* caterpillar.

Laboratory experiments

Performance tests: Conspecific comparison. Both *P. brassicae* and *M. brassicae* eggs hatched 5 days after oviposition. When eggs hatched, 10 neonates were left on the eggs(+) plant but transferred with a brush to the adaxial side of the same leaf. Another 10 neonates were transferred to the adaxial side of a leaf on an eggs(-) plant (Fig. 1). Caterpillar weight was measured 8 days after oviposition (3 days after hatching) on a microbalance (accuracy = 1 µg; Sartorius AG, Göttingen, Germany). After weighing, the caterpillars were transferred back to their original position on eggs(+) or eggs(-) plants. The same procedure was followed 12 days after oviposition (7 days after hatching). In total, 30 egg(+) plants (10 HR+, 10 HR- with *P. brassicae* eggs, 10 with *M. brassicae* eggs) and 30 eggs(-) plants (20 with *P. brassicae* and 10 with *M. brassicae* caterpillars) were used. In order to investigate if removal of excess eggs had an effect on the plants' response, an additional control experiment was conducted. All eggs of either *P. brassicae* or *M. brassicae* were removed from the eggs(+) plant after oviposition in the same way as described in Materials and methods: *Plant treatments*. Eggs(-) plants were used as controls. Additionally, oviposition was allowed to an extra plant. When caterpillars emerged from the eggs on this extra plant, 10 caterpillars were transferred to the abaxial side of the fourth or fifth leaf of the plant with the eggs experimentally removed, and 10 caterpillars were transferred to the abaxial side of the fourth or fifth leaf of the eggs(-) plant. Caterpillar mass was measured eight and 12 days after oviposition (three and seven days after hatching) on a microbalance (accuracy = 1µg; Sartorius AG, Göttingen, Germany). After weighing, the caterpillars were transferred back to their original position on eggs(+) or eggs(-) plants.

In total 30 plants were used for this control experiment for each herbivore: 10 eggs(+) plants with eggs removed, 10 eggs(-) clean control plants and 10 used as caterpillar-supplying plants.

Heterospecific comparison: We removed *P. brassicae* eggs just before hatching (5 days after oviposition) and inoculated the eggs(+) plants with 10 *M. brassicae* neonates as well as eggs(-) plants and *vice versa*. The mass of *M. brassicae* caterpillars that developed on eggs(+) plants (previously infested with *P. brassicae* eggs), was measured and compared with the weight of *M. brassicae* caterpillars that developed on eggs(-) plants and *vice versa* (Fig. 1). In total, 30 eggs(+) plants (10 HR+, 10 HR- with *P. brassicae* eggs, 10 with *M. brassicae* eggs) and 30 eggs(-) plants (20 with *M. brassicae* and 10 with *P. brassicae* caterpillars) were used.

Plant growth and flowering: Plant development was also evaluated. We calculated the difference in plant height between five and 12 days after oviposition by measuring the length of the main stem up to the apical tip of eggs(+) and egg(-) plants. The first measurement was taken at day 5 after oviposition, before caterpillars hatched from the eggs, and the second measurement was at day 12 after oviposition, when caterpillars had fed on the plants for 7 days. Moreover, we measured the number of plants that had initiated flowering on day 5 after oviposition, before eggs hatched.

Semi-field experiment

The semi-field experiment was replicated three consecutive times in May and June 2011 in a common garden field plot at the experimental farm (Unifarm) of Wageningen University, Wageningen, The Netherlands. (Because plants were kept in the laboratory for the first five days after infestation and were moved to the field after eggs hatched, we call this a “semi-field experiment”). Each replicate consisted of 20 *B. nigra* plants, 10 eggs(+) plants infested with

P. brassicae eggs, and 10 eggs(-) plants used as a control. Four-week-old non-flowering plants were used for the experiments. Plants were kept in a climate chamber (21 ± 2ºC, L16:D8, 83 µmol m^{-2} s^{-1} PAR) until 5 days after oviposition. When eggs hatched, neonates were transferred to eggs(+) or eggs(-) plants as described in Materials and methods: *Plant treatments*. Subsequently, eggs(+) and eggs(-) plants were moved from the climate chamber to the field. Each experimental trial consisted of five plots. Using a randomized design, each of the 5 plots (50 cm x 50 cm) contained four *B. nigra* plants, two eggs(+) and two eggs(-) plants. The distance between plots was 1 m. Caterpillar mass and number of flowering plants, were measured as described in Materials and methods: *Conspecific comparison*. Plant height was measured only at day 12 after oviposition.

Statistics

Laboratory experiment

The mass of caterpillars that developed on eggs(+) and eggs(-) plants was compared using a Wilcoxon signed-rank test. The effects of the eggs and caterpillars of *P. brassicae* and *M. brassicae* on plant growth between five and 12 days after oviposition were analysed with a general linear model and were treated as fixed effects. Assumptions of normality and homoscedasticity of the error term were tested and satisfied

$$y= \beta_0+\beta_1 x_{eM} +\beta_2 x_{eP}+\beta_3 x_{cP} +\beta_4 x_{eM*cP}+\beta_5 x_{eP*cP}+ \varepsilon$$

where y is the difference in plant height between day 5 and day 12, β_0 is the effect on plant height of *M. brassicae* caterpillars on plants without eggs, $\beta_1 x_{eM}$ is the effect of *M. brassicae* eggs on plant height, $\beta_2 x_{eP}$ is the effect of *P. brassicae* eggs on plant height, $\beta_3 x_{cP}$ is the effect of *P. brassicae* eggs on plant height, $\beta_4 x_{eM*cP}$ is the effect of *M. brassicae* eggs and *P. brassicae* caterpillars on plant height, $\beta_5 x_{eP*cP}$ is the effect of *P. brassicae* eggs and *P. brassicae* caterpillars on plant height, ε is the random error term. The effect of eggs on plant developmental phase on day 5 after egg deposition was analysed using logistic regression. Vegetative phase i.e. plants without open flowers and reproductive phase i.e. plants with open flowers were used as binary dependent variables. Eggs were used as a fixed effect, and the model followed a binomial distribution. Statistical analysis for all laboratory experiments was conducted using R software version 2.13.1 (R Development Core Team 2008).

Semi-field experiment

The mass of caterpillars that developed on eggs(+) and eggs(-) plants was compared using a Wilcoxon signed-rank test. The plant height of eggs(+) and eggs(-) plants was compared using a Wilcoxon signed-rank test. A chi-square goodness-of-fit test was used to compare proportion of flowering plants between eggs(+) and eggs(-) plants. Statistical analysis for the semi-field experiments was conducted using R software version 2.13.1 (R Development Core Team 2008).

Results

Laboratory experiments

Plant-mediated effects of *P. brassicae* oviposition on caterpillar performance

Conspecific comparison: *Pieris brassicae* caterpillars that developed on eggs(+) HR+ plants, gained significantly less mass than those developing on eggs(-) plants at eight and 12 days after oviposition (both: $P < 0.001$, Fig. 2, Table S1, Appendices). Similar effects were recorded on eggs(+) HR- plants when compared with eggs(-) plants (day 8: $P < 0.001$, day 12: $P < 0.001$, Fig. 2, Table S1, Appendices). There was no plant-mediated effect on caterpillar weight when all eggs had been removed immediately after egg deposition (*P. brassicae*: day 8: $P = 0.515$; day 12: $P = 0.929$, Table S2, Appendices). Moreover, no necrotic spots were observed on any of the eggs(+) plants in this control experiment.

Heterospecific comparison: *Mamestra brassicae* caterpillars gained significantly less mass when developing on plants previously infested with *P. brassicae* eggs than those developing on eggs(-) plants eight and 12 days after oviposition, irrespective of HR (all comparisons: $P < 0.001$, Fig. 2, Table S1, Appendices).

Plant-mediated effects of *M. brassicae* moth oviposition on caterpillar performance

Conspecific comparison: At day 8 and 12 after oviposition, there was no plant-mediated effect of *M. brassicae* egg deposition on mass of *M. brassicae* caterpillars (day 8: $P = 0.52$; day 12: $P = 0.53$, Fig. 3, Table S1, Appendices). When all eggs were removed from eggs(+) plants early in the morning following oviposition, no effect on caterpillar weight was observed (day 8: $P = 0.268$; day 12: $P = 0.158$, Table S2, Appendices).

Heterospecific comparison: There was no plant-mediated effect of *M. brassicae* egg deposition on mass of *P. brassicae* caterpillars, neither at day 8 nor at day 12 after oviposition (8 d: $P = 0.30$; 12 d: $P = 0.13$, Fig. 3, Table S1, Appendices).

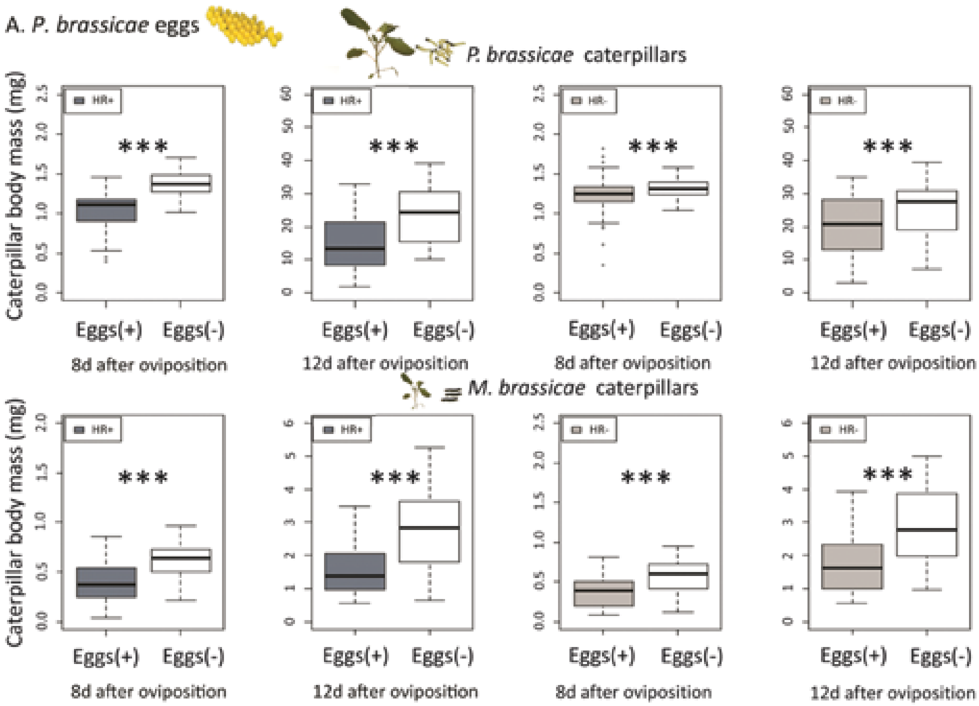

Figure 2. Plant-mediated effects of *P. brassicae* oviposition on caterpillar performance. Body mass (mg) of caterpillars that developed from neonates to 2nd larval instars on *B. nigra* plants previously infested with eggs (eggs(+)), compared to caterpillars developing on egg-free plants (eggs(-)). Number of eggs(+) (HR+/HR-) and eggs(-) plants= 80, ***$P < 0.001$, (Wilcoxon signed-rank test).

Effects of oviposition on plant growth and flowering

Plant growth was significantly affected by the presence of *P. brassicae* eggs ($F_{2,113}=22.852, P<0.001$, Fig. 4a) but following egg hatch, feeding by either *P. brassicae* or *M. brassicae* caterpillars did not affect plant growth ($F_{1,112} = 2.93$, $P = 0.09$, Table S3, Appendices). Plants exposed to *P. brassicae* eggs grew taller ($t = 3.09$, $P = 0.003$, Fig. 4a, Table S3, Appendices) compared with eggs(-) plants. *Mamestra brassicae* egg deposition, however, did not affect plant growth ($t = -1.24$, $P = 0.22$, Fig. 4a, Table S3, Appendices). There was no interaction effect of eggs and caterpillars on plant growth ($F_{2,\ 110} = 1.074$; $P = 0.35$, Fig. 4a, Table S3, Appendices). Plants bloomed sooner: a larger proportion of plants flowered five days after oviposition by *P. brassicae* compared to control plants ($z = 3.263$, $P = 0.001$, Fig. 4b, Table S4, Appendices). *Mamestra brassicae* eggs did not affect flowering time ($z = 0.230$, $P = 0.82$, Fig. 4, Table S4, Appendices).

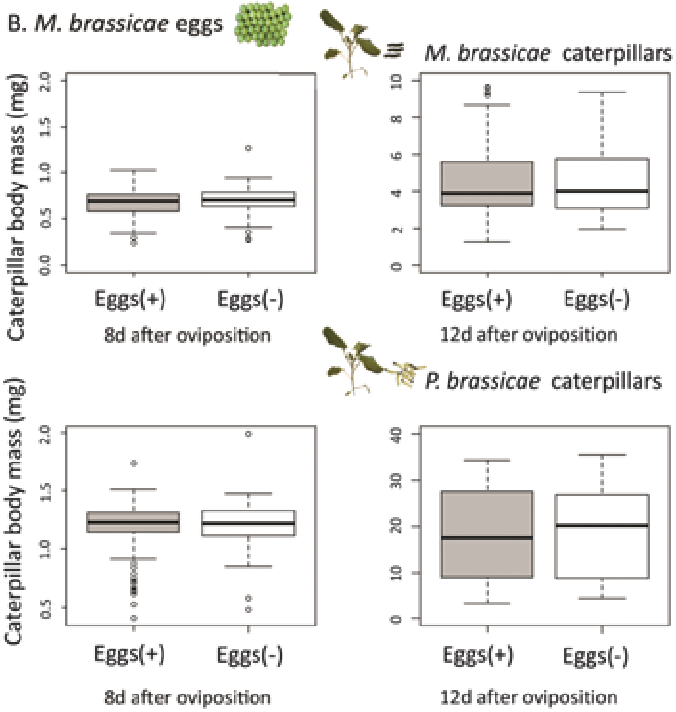

Figure 3. Plant-mediated effects of *M. brassicae* oviposition on caterpillar performance. Body mass (mg) of caterpillars that developed from neonates to 2nd larval instars on *B. nigra* plants previously infested with eggs (eggs(+)), compared to caterpillars developing on egg-free plants (eggs(-)). Number of eggs(+) and eggs(-) plants = 40, (Wilcoxon signed-rank test).

Semi-field experiment

Plant-mediated effects of *P. brassicae* oviposition on caterpillar performance

Pieris brassicae caterpillars developing on eggs(+) plants expressing HR gained significantly less biomass compared to caterpillars developing on eggs(-) plants eight and 12 days after oviposition (both: $P < 0.001$, Fig. 5). Similar effects were recorded for HR- plants (day 8, $P < 0.001$; day 12: $P = 0.03$, Fig. 5).

Effects of oviposition on plant growth and flowering

Pieris brassicae eggs(+) plants were significantly taller 12 days after oviposition compared with eggs(-) plants regardless of the expression of HR (HR+: $P = 0.019$; HR-: $P = 0.031$, Fig. 6). Significant differences were found in flowering time between eggs(+) and eggs(-) plants five days after oviposition by *P. brassicae* regardless of HR (HR+: $\chi^2 = 5.167$, $P = 0.011$; HR-: $\chi^2 = 6.805$, $P = 0.004$, Fig. 6).

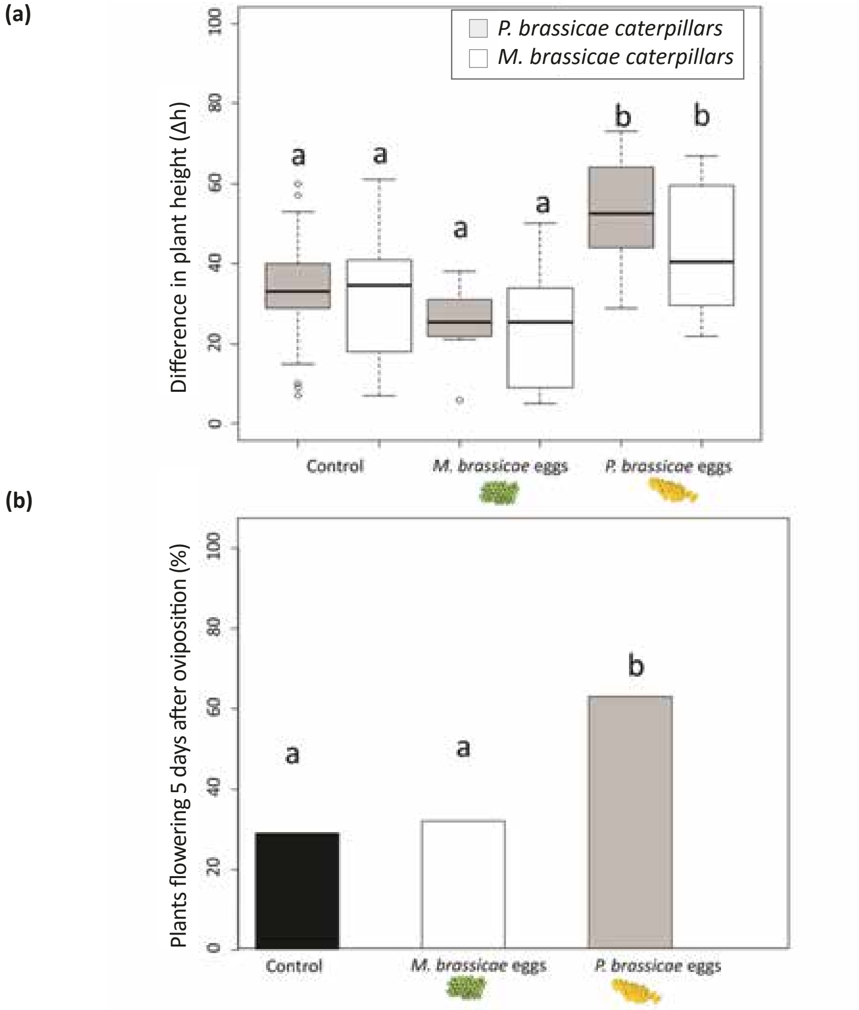

Figure 4. Effects of oviposition on plant development. (a) The difference in plant height (Δh) from day 5 (when eggs hatch) to day 12 (seven days of caterpillar feeding) on *B. nigra* plants previously infested with eggs [eggs(+)] compared to egg free *B. nigra* plants [eggs(-)]. The height of the boxes represent the first to the third quartile of the range, the horizontal line within the box is the median, the whiskers indicate the data minimum and maximum and the open circles represent outliers. (b) The effect of oviposition on plant developmental phase at day 5 since oviposition (when eggs hatch). Number of plants exposed to eggs and egg free plants, n= 120. Different lowercase letters above boxplots indicate significant differences among treatments. $P < 0.05$, (GLM).

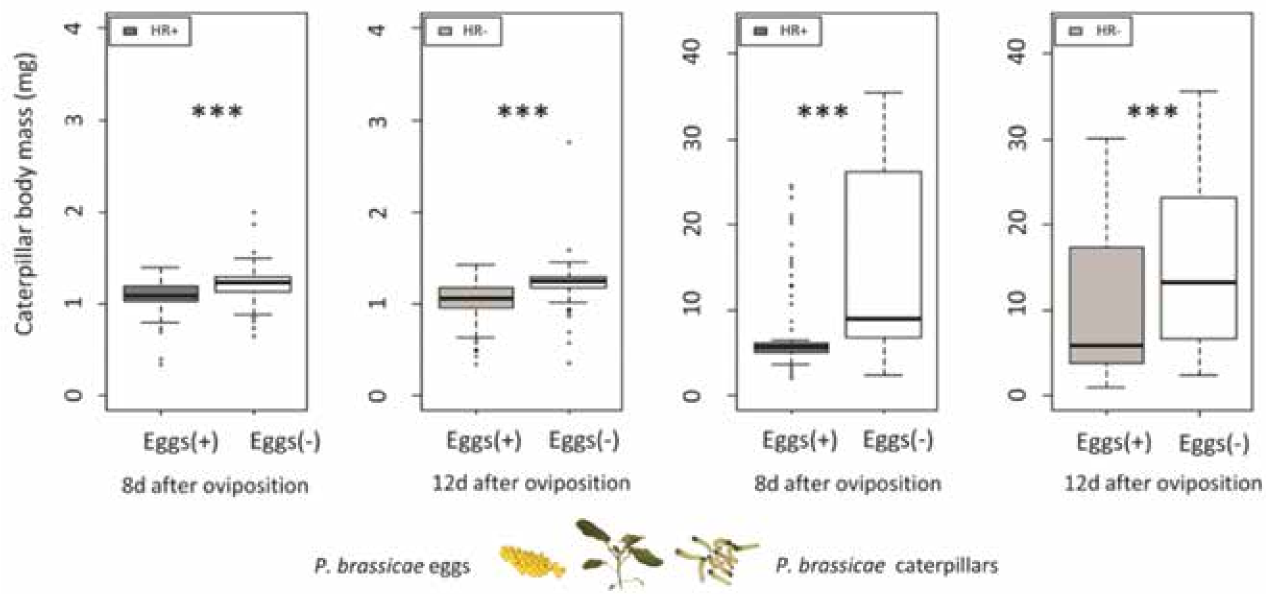

Figure 5. Plant-mediated effects of *P. brassicae* oviposition on caterpillar performance under semi-field conditions. Body mass (mg) of *P. brassicae* caterpillars that developed from neonates to 2nd larval instars on plants exposed to eggs (eggs(+)) compared to *P. brassicae* caterpillars that developed on egg-free plants (eggs(-)). Number of eggs(+) (HR+/HR-) and eggs(-) plants = 60, ***$P < 0.001$, (Wilcoxon signed-rank test).

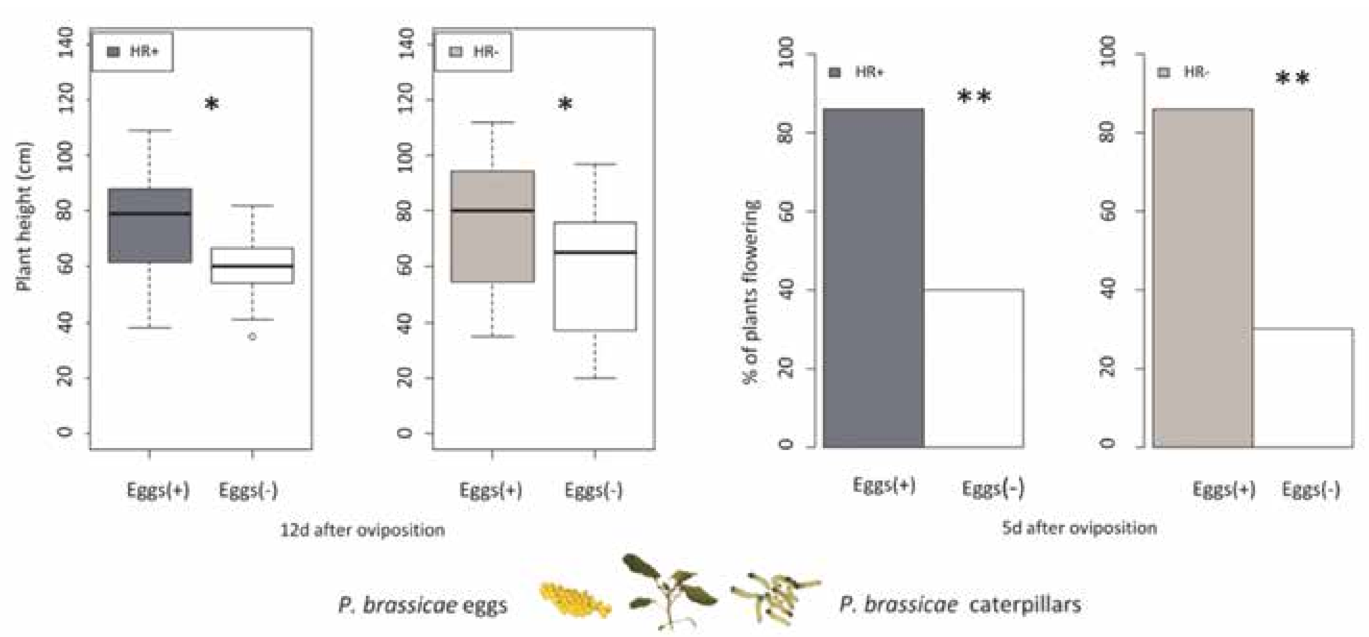

Figure 6. Plant height (cm) of eggs(+) *B. nigra* 12 days after infestation, compared to eggs(-) plants. The height of the boxes represents the first to the third quartile of the interquartile range; the horizontal line within the box is the median; and the whiskers are the data minima and maxima. Open circles represent outliers. *$P < 0.05$ (Wilcoxon signed-rank test). Plant proportion of flowering *B. nigra* eggs(+) plants (%) is shown, 5 days after *P. brassicae* oviposition, compared to eggs(-) plants. Number of eggs(+) (HR+/HR-) and eggs(-) plants = 60, *$P< 0.05$, **$P < 0.01$, (χ^2-test, goodness of fit).

Discussion

Our results show that egg deposition by *P. brassicae* butterflies affects the performance of subsequently feeding larvae of conspecifics and *M. brassicae* and enhances plant growth and reproduction. In contrast, egg deposition by *M. brassicae* moths does not. Thus, we demonstrate that plant resistance induced by exposure to eggs of *P. brassicae* differs from the plant response induced by eggs of *M. brassicae* (specificity of induction). *Brassica nigra* responds to *P. brassicae* eggs with a clear necrosis-resembling hypersensitive response, which can lead to high *Pieris* egg mortalities in nature (Shapiro & DeVay 1987, Fatouros *et al.* 2012). Interestingly, no necrosis developed, when all *P. brassicae* eggs were removed from the plant shortly after oviposition. Yet, our results indicate that the hypersensitive response induced by *P. brassicae* eggs as such is unlikely to have a significant impact on later plant responses against feeding caterpillars: caterpillar weight was negatively affected by *P. brassicae* egg deposition regardless of HR expression.

Caterpillars that grow slowly and consequently have a longer developmental time have an extended window of vulnerability to natural enemies (Loader & Damman 1991, Benrey & Denno 1997, Mattiacci *et al.* 2001, Bukovinszky *et al.* 2009, Harvey & Gols 2011b). Such a plant-mediated response to egg deposition was not observed when *B. nigra* plants were exposed to eggs of the cabbage moth *M. brassicae*. Our results show that caterpillars that fed and developed on *M. brassicae* eggs(+) plants grew equally well as caterpillars that fed and developed on eggs(-) plants.

Specificity of plant resistance mediated by oviposition of two different herbivores

Specificity in induction mediated by *P. brassicae* oviposition can be explained by different reasons. First, differences in the mode of egg deposition or constituents in egg-associated secretions might cause the specificity of induction. Fatouros *et al.* (2012) revealed that volatiles of *B. nigra* plants induced by *P. brassicae* eggs attract egg and larval parasitoids, whereas volatiles induced by *M. brassicae* eggs did not. Electron microscopic analysis revealed structural differences at the plant-egg interface: *Pieris brassicae* tightly glues the eggs on the *B. nigra* surface, whereas *M. brassicae* eggs can be removed easily (Fig. 7).

Indeed, how firmly eggs are attached to the leaf surface may have an effect on different cells that are able to perceive information about when an egg has been laid (Hilker & Meiners 2006). Oviduct secretion used by female herbivores to glue eggs onto plant tissue has been shown to contain the elicitor affecting the transcriptomic profile of the attacked plant (Doss *et al.* 1995, Doss *et al.* 2000, Hilker *et al.* 2005, Fatouros *et al.* 2008a, Köpke *et al.* 2010). Secondly, the specificity in the induction may be due to variation in herbivore tolerance and detoxification ability. *Brassica nigra* is a natural host plant of *P. brassicae* and probably co-evolved with this abundant butterfly (Harvey *et al.* 2007, Blatt *et al.* 2008). *Pieris brassicae* larvae are voracious and prefer to feed on *B. nigra* flowers during the second and subsequent instars and effectively detoxify glucosinolates that occur in five-fold higher levels in flowers than in leaves. Florivory by *P. brassicae* might act as a strong selection pressure on *B. nigra* plants (Smallegange *et al.* 2007). A recent review by Ali & Agrawal (2012) indicates that plants can recognize particularly those specialist herbivores that have high impact on plant fitness and respond accordingly.

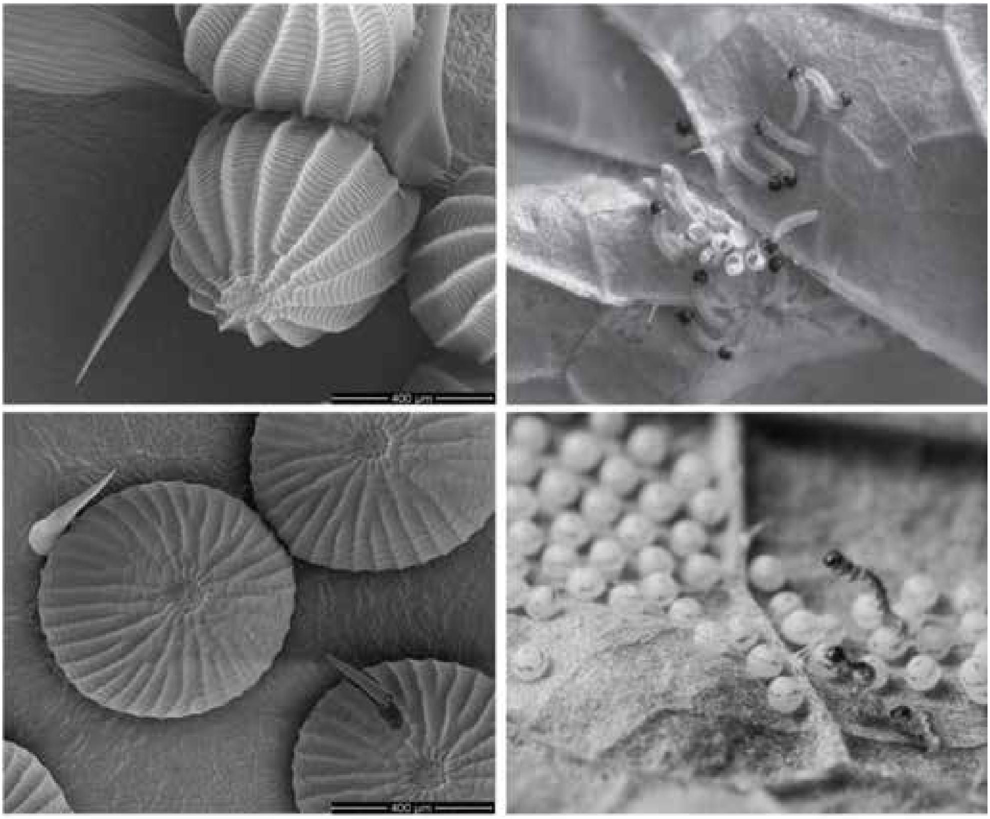

Figure 7. Eggs (left) and neonate larvae (right) of the two studied herbivores on *Brassica nigra*: the specialist *Pieris brassicae* (top) and the generalist *Mamestra brassicae* (bottom).

It is likely, therefore, that *B. nigra* plants evolved to respond to eggs of the florivorous *P. brassicae,* before the caterpillars hatch and start feeding on leaves and flowers. Plants benefit from low induction when attacked by sequestering specialists (indirect defences are not effective in strong induction, because sequestering specialists use the plant toxins for their own benefit against their natural enemies) and strong induction when attacked by non-sequestering specialists (plants benefit from indirect defences). Our results indicate that plant-mediated effects of *P. brassicae* oviposition are strong since there is a clear effect on herbivore performance. Moreover, Fatouros *et al.* (2012) showed that volatiles of *B. nigra* plants induced by *P. brassicae* egg deposition attract egg and larval parasitoids, thus *B. nigra* probably also benefits from indirect defence. There is less selection pressure, however, on *B. nigra* to respond to the *M. brassicae* larvae, which exclusively feed on leaves and show poor performance on wild crucifers with high levels of aliphatic glucosinolates such as *B. nigra* (Gols *et al.* 2008, Harvey *et al.* 2010) and tend to leave those plants quickly after egg hatch (Johansen 1997; F.G. Pashalidou, *personal observations*).

Therefore, it is likely that *M. brassicae* does not circumvent plant recognition because there is no need for the plant to defend or recognize its eggs. Moreover, herbivore abundances might play a role in the evolution of plant defences against insect eggs. Oviposition by the abundant pine sawflies *Diprion pini* and *Neodiprion sertifer,* was shown to induce the emission of pine volatiles on *Pinus sylvestris*, which results in the attraction of egg parasitoids (Mumm *et al.* 2005). However, eggs of the less abundant pine sawfly *Gilpinia pallida* did not induce such a response. Thus, *P. sylvestris* might have evolved to specifically respond to eggs of its abundant enemy *D. pini* as a counter-adaptation to high levels of feeding damage (Mumm & Hilker 2005, 2006). Even though *P. brassicae* is a specialist and *M. brassicae* is a generalist herbivore on Brassicaceae, we cannot conclude from this study that diet breadth is the (only) explanation for specificity of induction. The comparison of specialists and generalists is a classical paradigm for studies on how insects interact with plants.

A recent review by Ali & Agrawal (2012), however, convincingly argues that none of the studies that compared more than two herbivores so far found a consistent pattern that specificity of induction was associated with diet specialization. Instead, available data suggest that different feeding guilds (leaf chewers vs. phloem feeders) consistently induce differently. To test whether specificity of plant responses to herbivore eggs is linked to diet breadth, future experiments should include more specialist and generalist lepidopteran herbivores. Different herbivores may alter the direction of selection pressure on major defensive compounds in *B. nigra*, showing that induced responses in this plant can be plastic depending on the particular community context in which they grow. Cues from predators and parasites, moreover, can cause rapid evolution of induced defensive traits in their hosts or prey via phenotypic plasticity, not only in terrestrial but also in aquatic systems (Thompson *et al.* 1999, Tollrian & Harvell 1999, Agrawal *et al.* 2001, Peacor *et al.* 2011). Many planktonic organisms show anti-predator defence responses upon recognition of their enemy through chemical communication (Lass & Spaak 2011). Specificity of induced morphological changes has been shown in e.g. cladocerans, protozoans, and barnacles (Kuhlman & Heckmann 1985, Dodson 1989, Tollrian & Dodson 1999). For example, cladocerans of the genus *Daphnia* induce morphological defences, i.e. helmet formation, in the presence of specific predator kairomones (Tollrian & von Elert 1994) and marine planktonic crustaceans reduce their foraging time and feeding behaviour in the presence of specific predator kairomones in order to reduce their visibility (Lass & Spaak 2011). Overall, specificity of induced resistance via adaptive phenotypic plasticity of individuals can influence complex ecological community structures.

Plant-mediated effects of insect egg deposition: priming or suppression?

It has been demonstrated that the immune system of plants can be primed in response to specific environmental signals, to get “ready for the battle” (Conrath *et al.* 2006, Frost *et al.* 2008). When primed plants are subsequently attacked, they respond more rapidly and more strongly than non-primed plants to the environmental signal. Priming is assumed to impose lower costs for a plant than an induced resistance response (Frost *et al.* 2008). Environmental signals such as herbivore-induced plant volatiles in maize not only prime the production of HIPVs in undamaged plants and the attraction of parasitoids to primed plants, but on primed plants *Spodoptera littoralis* growth rate was also lower compared to that on untreated control plants (Ton *et al.* 2007). Thus, any reliable environmental signal that indicates herbivory can serve as a cue that induces priming (Frost *et al.* 2008), including insect egg deposition (Frost *et al.* 2008, Dicke 2009, Hilker & Meiners 2011).

Plants might recognize herbivore eggs as a signal that is associated with future herbivory and mobilize defences to be prepared against the newly hatched larvae. Just recently, Kim *et al.* (2012) showed that tomato plants recognize oviposition by *Helicoverpa zea* moths as a signal for future herbivory, induction of a gene encoding a protease inhibitor (*Pin2*) and the accumulation of the phytohormone jasmonic acid (JA), both involved in anti-herbivore defences, were primed by *H. zea* egg deposition followed by simulated *H. zea* herbivory. Egg deposition by the pine sawfly may “warn” pine trees for future feeding by sawfly larvae. *Diprion pini* larvae which fed on *P. sylvestris* twigs that were previously infested with eggs of conspecifics, were lighter compared to larvae that developed on egg-free twigs (Beyaert *et al.* 2011). The resistance induced by oviposition resulted in a reduced fecundity of the adult female sawflies of the next generation.

Moreover, transcript levels of sesquiterpene synthase genes *Ps*TSP1 and *Ps*TSP2 were increased on twigs exposed to eggs compared to egg-free twigs and decreased when larvae started feeding.

On the other hand, herbivores can adapt to plant responses and manipulate their food plant by suppressing plant defences (Bruce & Pickett 2007, Bruessow *et al.* 2010). Especially generalist herbivores, which are typically more sensitive to specific plant toxins than specialists, are expected to suppress induced plant responses (Ali & Agrawal 2012). *Arabidopsis thaliana* plants that were treated with an extract of *P. brassicae* eggs were more susceptible to caterpillars of the generalist moth *Spodoptera littoralis* because of the suppression of plant defence. There was no effect, however, on larval growth of the specialist *P. brassicae* (Bruessow *et al.* 2010). The phytohormone salicylic acid (SA) was shown to accumulate at the oviposition site and negatively interfered with JA signalling. Thus, crosstalk between these two phytohormones, both known to be crucial signals in induced plant defences against herbivores (Reymond & Farmer 1998, Kessler & Baldwin 2002, Dicke *et al.* 2009, Pieterse *et al.* 2009), leads to suppression of plant defence. Bruessow *et al.* (2010) used egg extracts of *P. brassicae,* which were previously shown to elicit a transcriptional response similar to egg deposition (Little *et al.* 2007) in order to demonstrate the effects of eggs of herbivorous insects on plant defences.

Our data show that egg deposition by *P. brassicae* on *B. nigra* plants results in an enhanced rather than an attenuated resistance to *P. brassicae* and *M. brassicae* caterpillars. Pastor *et al.* (2012) proposed that in primed plants, inactive hormone conjugates could accumulate to higher levels in the vacuole, allowing for a faster and stronger release of the active metabolites upon pathogen/herbivore attack. We expect a similar priming mechanism as described in Kim *et al.* (2012); JA accumulation might be primed at the oviposition site upon subsequent herbivory, which leads to a stronger and faster induction of plant defence and reduction in larval mass. *Brassica nigra* is a common, natural host plant of *P. brassicae.* Its response to *P. brassicae* eggs in terms of enhanced resistance shows that this plant responds to the eggs of a voracious herbivorous insect in a way that reduces its quality for the herbivore.

Egg deposition by *P. brassicae* affects plant development

Interestingly, *P. brassicae* oviposition also affects plant growth and initiates early flowering in *B. nigra* plants. Plants carrying *P. brassicae* eggs flower faster before caterpillars hatch compared to eggs(-) plants and *M. brassicae* eggs(+) plants. Our results are in line with those from Lucas-Barbosa *et al.* (2012), who tested the effects of *P. brassicae* eggs on pollinator attraction and seed production of flowering *B. nigra* plants in a similar common garden experiment. They observed accelerated seed production in plants that had been exposed to eggs compared to uninfested plants. Moreover, plants started to produce seeds before caterpillars hatched and sooner than uninfested plants. Caterpillars did not feed on seeds and, thus, reproduction was safeguarded prior to the initiation of herbivory on flowers. Taking the two studies together, we suggest that oviposition by *P. brassicae* accelerates plant growth and reproduction. Many plant species exhibit tolerance to herbivory, i.e. they are able to compensate for feeding damage in various ways (Trumble *et al.* 1993, Strauss & Agrawal 1999). A study by Blatt *et al.* (2008) showed that *B. nigra* plants can tolerate herbivory by caterpillars of *P. brassicae* during the vegetative phase. Even though plants infested with larvae were smaller than control plants and flowered slightly later, seed production was not decreased. A similar effect was found when *B. nigra* was exposed to *Pieris rapae*, a lepidopteran species closely related to *P. brassicae*. Thus, the annual *B. nigra* appears to tolerate also a great degree of herbivory by two co-existing species of specialist butterflies.

Conclusions

Our findings show that *B. nigra* plants respond differently to egg deposition by two herbivores and develop resistance to subsequently feeding caterpillars only when exposed to egg deposition by *P. brassicae*. By prolonging larval development of the herbivore, and at the same time enhancing growth and reproduction, they safeguard offspring production. In that way, they can deal with the attack by *P. brassicae* caterpillars that switch from folivory to florivory within a few days after hatching. Egg deposition by *P. brassicae* induces resistance to herbivory in *B. nigra* in the laboratory and field and adds another level to adaptive plasticity in induced plant resistance starting at the initial phase of plant colonization before feeding damage has occurred.

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Chapter 3

Plant-mediated effects of butterfly egg deposition on subsequent caterpillar and pupal development, across different species of wild Brassicaceae

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Herbivory can change plant quality, which may have consequences for interactions between the inducing herbivore and other community members. Studies investigated effects of plant quality on herbivore performance, often neglected the egg stage, and instead introduced larvae onto the plant. Recently, we reported that herbivore oviposition by *Pieris brassicae* (Large Cabbage White butterfly) reduced plant quality of *Brassica nigra* (black mustard) for subsequent herbivores. It remains unclear how persistent and common these plant-mediated effects of oviposition are. Here, we used five species of wild Brassicaceae (*B. nigra, Brassica oleracea, Sinapis arvensis, Moricandia arvensis,* and *Moricandia moricandioides*). We determined the response to oviposition by the specialist *P. brassicae* following the natural sequence of events: oviposition, egg, larval, and pupal development. All tested plant species are known to interact with *P. brassicae* in nature. We assessed caterpillar, pupal mass, and development time on plants exposed to butterfly eggs compared to egg-free plants. We showed that plant-mediated effects of oviposition were not specific for *B. nigra* but occurred in all tested plant species except for *M. arvensis*. However, the strength of the plant-mediated effect on caterpillar growth depended on plant species and was more variable during early development of the caterpillars, but attained similar strength towards the pupal stage. Thus, across different members of the Brassicaceae family, oviposition can influence plant quality and has negative consequences on growth of the larvae, persisting until pupation. Further studies are needed to assess to what extent this trait might be phylogenetically conserved.

Keywords*:* Brassicaceae, egg deposition, subsequent herbivory, performance, specialist herbivore, variation

Introduction

Plant species and populations display variation in resistance traits, which mediate interactions with their insect communities (Lankau & Strauss 2007, Gols *et al.* 2009). These resistance traits, physical or/and chemical, are classified according to the timing of deployment into (a) traits that are *constitutively* produced, (b) traits that are *induced* upon herbivore attack or a combination of the two (Gatehouse 2002, Wu & Baldwin 2010). Induced plant responses are considered to reduce allocation costs as they are only employed when the plant is under attack (Poelman & Dicke, 2014).

For many insect-plant systems, it has been shown that induced plant responses change plant quality, impairing growth and survival of herbivores and hampering colonization by subsequently arriving herbivores (Karban & Baldwin 1997, Gols & Harvey 2008, Howe & Jander 2008). The term host-plant quality is used to describe the positive or negative effects of plant compounds (e.g. nitrogen, carbon levels, and defensive compounds) on herbivore performance (Awmack & Leather 2002). Induced plant responses can have consequences for successive interactions with herbivores and other species in the plant-associated community (Poelman *et al.* 2011, Kaplan & Denno 2007, van Zandt & Agrawal 2004, Viswanathan *et al.* 2005, Stam *et al.* 2014). These plant-mediated interactions can influence insects at different trophic levels associated with above or belowground tissues (van der Putten *et al.* 2001, Bezemer & van Dam 2005, Poelman *et al.* 2011, Erb *et al.* 2011). In addition herbivores of different feeding guilds (Soler *et al.* 2012, Kaplan & Denno 2007), and often herbivores' sequence of arrival can also induce plant changes (Poelman *et al.* 2008a, Poelman *et al.* 2008b, Erb *et al.* 2011). There is ample literature reporting that induced plant responses, both morphological and chemical, can be herbivore-species specific (Karban & Baldwin 1997, Agrawal 2000, van Zandt & Agrawal 2004, Viswanathan *et al.* 2005, Uesugi *et al.* 2013) and herbivores may respond differentially to these induced changes (Karban & Baldwin 1997).

Plants respond to egg deposition by herbivorous insects, which in most species precede larval feeding, by increasing defences that can impair egg development or even kill the eggs directly or indirectly by recruiting egg parasitoids (Hilker & Fatouros 2015).

These egg-killing defence mechanisms of plants (e.g. hypersensitive response (HR), necrotic lesions surrounding the eggs) have been described for more than 20 insect species in more than 20 different plant species (Hilker & Fatouros 2015). However, plant-mediated effects of egg deposition on subsequent herbivores during different developmental stages (i.e. from larvae to adults) and their interactions with other trophic levels have only recently been discovered in *Pinus sylvestris*, *Arabidopsis* *thaliana* and *Brassica nigra* (Pashalidou *et al.* 2013, Pashalidou *et al.* 2015, Beyaert *et al.* 2011, Geiselhardt *et al.* 2013, Bruessow *et al.* 2010, Hilfiker *et al.* 2014). Caterpillars of Cabbage White Butterflies, *Pieris spp.,* are voracious herbivores. The later stages of *P. brassicae* prefer to feed on the flowers of their brassicaceous host plants with potential negative consequences for plant fitness (Smallegange *et al.* 2007). Plants that respond to herbivores when these are still in the egg stage may, therefore, receive less herbivore damage and produce more flowers (Pashalidou *et al.* 2013) or produce seeds earlier (Lucas-Barbosa *et al.* 2012), i.e. they display a reproductive escape. However, it remains unknown whether plant-mediated effects of oviposition on subsequent herbivory are an intrinsic trait across different brassicaceous species.

The aim of this study was to explore whether herbivore egg deposition induces changes in plant quality that affect subsequent life stages of the herbivore (larvae and pupae) in different brassicaceous plant species. We selected the following five wild species: *Brassica oleracea* L., *B. nigra* L., *Sinapis arvensis* L., *Moricandia arvensis* L. and *M. moricandioides* Boiss. *Brassica oleracea* is a perennial plant with a glabrous waxy leaf surface and plant quality for insect herbivores varies among populations of this plant species (Gols *et al.* 2008). *Brassica nigra* and *S. arvensis* are annuals with non-waxy leaves that often carry trichomes. Both *Moricandia* species are annual plants with waxy leaves. All plant species used in this study are known to interact with *P. brassicae* in nature (Courtney & Chew 1987). *Pieris brassicae* is a gregarious herbivore, native in Europe and usually lays clutches of 40 or more eggs on the abaxial side of the leaves (Feltwell 1982). We compared herbivore development on plants infested with *P. brassicae* eggs that were allowed to develop into larvae, with plants, where neonate larvae of *P. brassicae* were introduced.

As proxies for herbivore fitness, we measured larval and pupal biomass and egg-to-pupal development time.

Materials & Methods

Plants and insects

We used two populations of *Brassica oleracea*, *Sinapis arvensis*, and *B. nigra*, respectively, and single populations of *Moricandia moricandioides* and *M. arvensis*. *Brassica nigra* seeds were collected from naturally growing populations at two different locations about 8 km apart, one near Heteren (coordinates: 51.951N, 5.7083E) and one near Wageningen (51.9666N, 5.6666E), The Netherlands. Seeds of *S. arvensis* were collected from two naturally growing wild populations, one on the island of Vlieland (53.3N, 5.0666E) in the northern part of The Netherlands and one from Buren (53.4472N, 5.7988E), located in the riverine area of The

Netherlands. Seeds of two wild populations of *B. oleracea* were collected along the South coast of England, to the West and North of Swanage, Dorset at locations that are known as Kimmeridge (50.617N, -2.118E) and Winspit (50.5848N, -2.0342E). *Moricandia moricandioides* and *M. arvensis* grow in arid habitats. Seeds were collected in Albacete province, South-East Spain. Two cohorts of 15 plants of the above-described species and populations were grown in successive weeks starting at the end of February until the last week of April 2012.

Seeds were germinated and the seedlings were transferred to pots filled with potting soil. Plants were grown in a greenhouse (18 ± 4 ˚C, 60-80% r.h., L16:D8) and were watered daily. *Brassica oleracea* plants, which grow more slowly than the other four plant species, were 5 weeks old, whereas the other plant species were 4 weeks old at the beginning of the experiments. The butterflies were from our laboratory colony initially collected from Brussels sprouts plants (*Brassica oleracea* var. *gemmifera*). *Pieris brassicae* was reared in a climatised room (22 ± 1°C, 50-70% r.h., L16:D8) on Brussels sprouts plants, *B. oleracea* var. *gemmifera* cv. Cyrus.

Plant treatments

Butterflies of *P. brassicae* were allowed to lay eggs on half of the experimental plants. Plants from each tested population or species were individually placed in a rearing cage with approximately 100 butterflies. Oviposition was carefully observed and the number of eggs laid on the plants were counted. When females had laid approximately 20 eggs, the plant (eggs(+): plant exposed to oviposition) was removed from the cage. Additional eggs, if any, were removed gently with a fine brush within 20 min following oviposition. A previous study has shown that the eggs and not airborne signals, scales or any other source from the butterflies induced the plants and affected caterpillar development, as larval growth was not affected when all eggs had been removed immediately after oviposition (Pashalidou *et al.* 2013). Plants that had no contact with butterflies or any other insect were used as a control (eggs(-) plants: plant not exposed to oviposition). All plants were kept in a greenhouse (18 ± 4˚C, 60-80% r.h., L16:D8). In total, 30 plants were used per population or species (15 eggs(+)- and 15 eggs(-) plants), apart from *M. arvensis* where 16 plants were used due to low germination rate (8 (eggs(+)- and 8 (eggs(-) plants). Plants were visually inspected for a hypersensitive response (HR)-like necrosis to eggs three days after oviposition and the percentage of plants expressing HR was calculated.

Plants were treated as previously described in Pashalidou *et al.* (2013). Briefly, when *P. brassicae* caterpillars emerged from eggs on an eggs(+) plant, 10 neonates were transferred with a fine brush to the adaxial side of the same leaf. Another 10 caterpillars were transferred to the adaxial leaf-side of an eggs(-) plant. Each caterpillar from each plant was weighed 7d after hatching on a microbalance (accuracy = 1 µg) (Sartorius AG, Göttingen, Germany). Because *P. brassicae* caterpillars can consume the entire plant in a few days depending on the size of the plant and caterpillar density, we transferred only 3 caterpillars back to the same plants after the 1st biomass measurement (seven days after hatching). Caterpillars were allowed to move and feed freely on the plants. As caterpillars tend to disperse in the final larval stage to find a site for pupation, plants were covered with fine nets five days before pupation. Larvae were inspected regularly and when they pupated, the date of pupation was recorded and pupae were weighed on an analytical balance (accuracy 0.1 mg, Mettler Toledo) one day later to allow the pupal cuticle to sclerotise.

As fitness proxies we compared (a) biomass of *P. brassicae* caterpillars 7 days after hatching, (b) pupal biomass, and (c) the development time from egg to pupa between the different plant populations and across species both on eggs(+) and eggs(-) plants.

Statistics

The performance data, larval and pupal mass and developmental time respectively, were statistically analysed using a linear mixed model (LMM):

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + d_{ij} + e_{ijk}$$

Where Y_{ijk} is the observed value (Y_{ijk}: caterpillar mass, pupal mass, or development time), μ is the general mean, α_i the fixed effect of the plant species, level i (i = *B. nigra, S. arvensis*, *B. oleracea*, *M. arvensis*, *M. moricandioides*), β_j the fixed effect of plant treatment, (j =eggs(+), eggs(-)) and $\alpha\beta_{ij}$ the interaction term. The two random terms in the model correspond to variation among populations nested within plant species d_{ij} (σ_d^2) and the variation among caterpillars developing on a single plant (= biological replicate) e_{ijk} (σ_e^2). An additional linear mixed model (LMM) analysis was performed on the insect performance data obtained for each of the five tested plant species to test for the effect of egg induction within each plant species. Plant treatment was entered as a fixed model factor, whereas population (*B. nigra*, *B. oleracea S. arvensis*) and plant individual were entered as a random factors. Model fitting was done by employing restricted maximum likelihood (REML) and statistical tests for fixed effects were based on approximated F-tests. Normality, independence, and homogeneity of variance were checked by inspection of the residuals after fitting the model. The data on *B. nigra* were subjected to an additional analysis to determine the effect of the HR-like response. We used a similar statistical model as described above in which we included an additional fixed term for HR-like response (yes/no) and removed the plant species term. All analyses were conducted using the R software version 2.13.1 and lme4 package (R Development Core Team 2008).

Results

Egg-induced hypersensitive response (HR)

Pieris brassicae eggs induced HR-like necrosis in both plant species with non-waxy pubescent leaf surfaces (*B. nigra* and *S. arvensis*), whereas it was weak or not observed in plant species with glabrous waxy surfaces (*B. oleracea* and *Moricandia* spp.). HR expression was the strongest in *B. nigra* where 47-73 % of the plants expressed HR (Table 1).

Table 1. Percentage of plants expressing HR-like necrosis induced by *P. brassicae* oviposition in five brassicaceous plant species.

Plant species	Population	HR (%)	N tested
Sinapis arvensis	Vlieland	20	15
	Buren	27	15
Brassica nigra	Heteren	73	15
	Wageningen	47	15
Brassica oleracea	Kimmberidge	7	15
	Winspit	0	15
Moricandia arvensis		0	8
Moricandia moricandioides		0	15

Effects on larval biomass

Egg induction ($F_{1,4}$ = 63.4, $P < 0.001$), plant species ($F_{1,4}$ = 13.8, $P < 0.001$), and their interaction ($F_{1,4}$ = 9.05, $P < 0.001$) had a significant effect on larval biomass. Egg induction reduced food quality in all plant species but not to the same extent (Fig. 1a, Table S1, Table S2, Appendices). The effect of egg induction was highly significant in *M. moricandioides, B. nigra*, and, *B. oleracea,* almost significant in *S. arvensis* ($F_{1,4}$= 3.54, P= 0.06, Table S2, Appendices) and not significant in *M. arvensis* ($F_{1,4}$= 0.31, P = 0.58, Table S2, Appendices). Moreover, plant species itself also influenced larval mass; caterpillar performance was higher on *B. nigra* plants compared to *B. oleracea* and *M. arvensis,* and *S. arvensis* (Fig. 1a, Table S2, Appendices). In *B. nigra,* egg-induced HR had no subsequent effect on larval mass ($F_{1, 1}$ = 0. 19, P = 0.65).

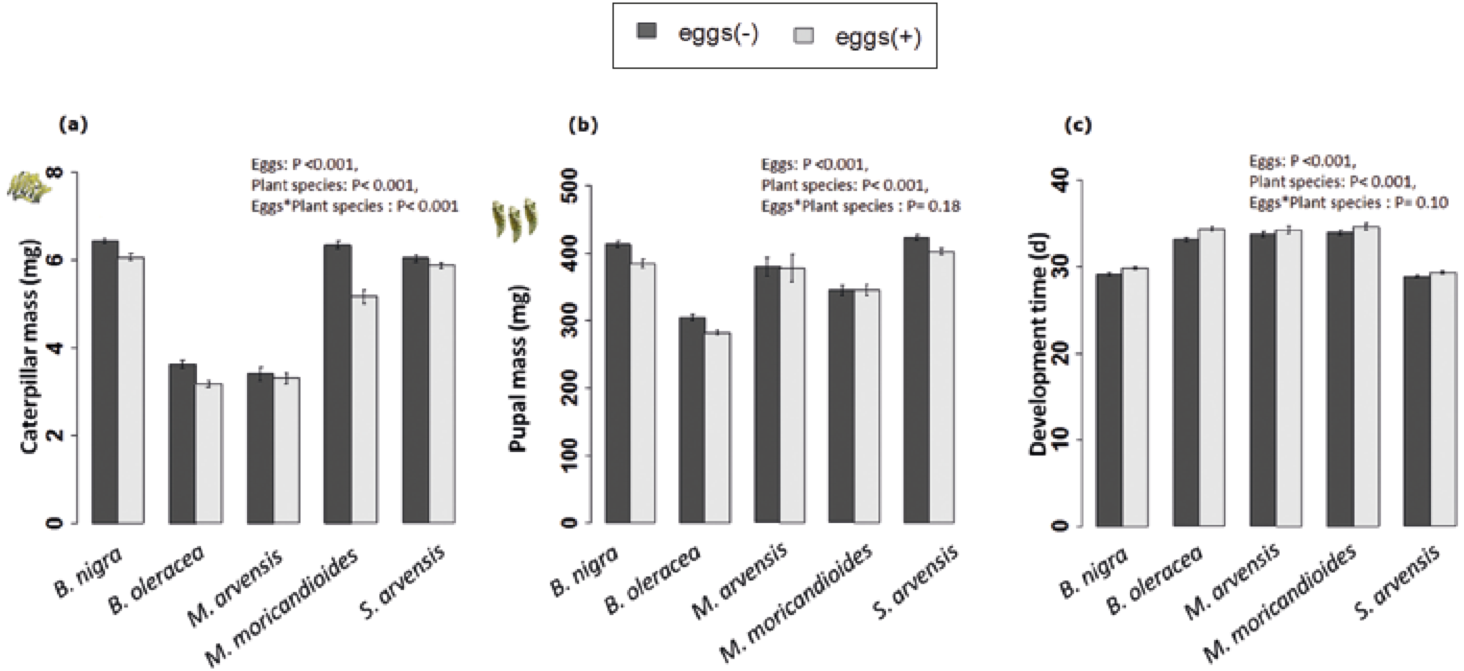

Figure 1. Plant-mediated effects of *Pieris brassicae* oviposition on the performance of larvae and pupae. Bars (mean ± SE) indicate the body mass (mg) of *P. brassicae* (a) 7day-old caterpillars, (b) pupae, and (c) egg-to-pupa development time (days) on different plant species *(B. nigra, B. oleracea, S. arvensis, M. arvensis, M. moricandioides*). *Pieris brassicae* developed on plants previously infested with eggs (eggs(+): light grey bars) or on egg-free plants (eggs(-): dark grey bars); the statistical results refer to general linear mixed model analysis that are further explained in the Results section.

Effects on pupal biomass

Egg induction resulted in a lower pupal mass ($F_{1,\ 4}$ = 26.76, *P* < 0.001, Fig. 1b, Table S3, Appendices). The interaction between treatment and plant species was not significant ($F_{1,4}$ = 1.54, *P* = 0.18). Only when the effect of egg-induction was analysed for each of the species separately, species-specific effects were revealed, egg induction affected pupal mass in *B. nigra*, *B. oleracea,* and *S. arvensis,* but not in *M. moricandioides* and *M. arvensis* (Table S3, Appendices). Moreover, plant species itself also influenced pupal mass; which was higher on *S. arvensis* and *B. nigra* plants compared to *M. arvensis*, *M. moricandioides* and *B. oleracea* (Fig. 1b, Table S3, Appendices). Egg-induced HR-like necrosis had no effect on pupal mass of *P. brassicae* that had been feeding on eggs(+) *B. nigra* ($F_{1,\ 1}$ = 2.85, *P* = 0.09).

Effects on egg to pupa development time

Egg induction extended larva-to-pupa development time of *P. brassicae* feeding on these plants ($F_{1,\ 4}$= 28.81, *P* < 0.001, Fig. 1c, Table S4, Appendices). The interaction between development time and treatment was not significant ($F_{1,\ 4}$ = 0.99, *P* = 0.41). Similar as for pupal mass, species-specific effects were only revealed when statistical analyses were performed for each plant species separately; egg induction affected development time in *B. nigra*, *B. oleracea,* and *S. arvensis,* but not in the two *Moricandia* species (Table S4, Appendices). Moreover, plant species itself also influenced development time; which was longer on *M. arvensis*, and *M. moricandioides* compared to *S. arvensis* and *B. nigra* and *B. oleracea* (Fig. 1c, Table S4, Appendices). HR had no effect on egg-to-pupa development time of *P. brassicae* growing on *B. nigra* ($F_{1,\ 1}$= 2.63, *P* = 0.10).

Discussion

Our results support previous findings that early non-feeding life stages of herbivores such as eggs can change plant quality. Egg-induced negative changes in plant quality were shown in most of the tested host-plant species and these effects were most pronounced during early larval development, but still, albeit to a lesser extent, affected pupal mass and larva-to-pupa development time.

Thus, we show that this plant-mediated negative effect of egg deposition is not restricted to *B. nigra* (Pashalidou *et al* 2013), but is also found in other wild brassicaceous species.

Our results corroborate those of Geiselhardt *et al.* (2013), who investigated the effects of egg induction on *Arabidopsis thaliana*, on subsequent development of *P. brassicae* caterpillars. Geiselhardt *et al.* (2013) found that caterpillars caused less feeding damage, weighed less, and suffered twice the level of mortality on plants previously infested with eggs, compared to caterpillars that developed on egg-free plants. Bruessow *et al.* (2010) have described contrasting results, where application of *P. brassicae* egg extract on *A. thaliana* had no effect on the biomass of *P. brassicae* caterpillars. Treatment with egg extract followed by caterpillar feeding supressed the expression of genes that were induced in control plants not treated with the extract (Bruessow *et al.* 2010). It has also been shown that egg deposition causes priming, i.e. the plants activate their responses more rapidly in response to a new stress, e.g. feeding by caterpillars (Kim *et al.* 2012). Kim *et al.* (2012) reported priming in tomato plants in terms of induction of jasmonic acid, a phytohormone playing an important role in the regulation of induced plant defences, with a concomitant increase of *PIN1* gene transcripts. It remains to be elucidated which mechanisms underlie the egg-induced resistance effects reported here and whether the same mechanisms are triggered in each of the plant species.

In this study, we have compared the effect of induction by *P. brassicae* eggs on plant quality in several wild plant species belonging to the Brassicaceae to reveal if these effects are common within this plant family. Our results show that the plant species differ in the extent to which egg induction affects plant quality and the subsequent development of the caterpillar and pupal stages. There were no plant-mediated effects on *P. brassicae* when developing on *M. arvensis* and only effects on early larval development when caterpillars were feeding on egg-induced *M. moricandioides* plants (based on within-species statistical analyses). A plausible explanation for these results could that because *M. arvensis* and *M. moricandioides* represent host plants of poorer quality for *P. brassicae* compared to e.g. *B. nigra* and *S. arvensis*. Thus, this low plant quality may overrule the potential effect of egg induction.

Moreover, a recent study reported that the main herbivores of *Moricandia* species are large grazers such as sheep and goats (Gómez 1996). Though *P. brassicae* is also a common insect herbivore on these species, Gómez (1996) claimed that the defoliation of the plants due to the grazers is so strong that any potential selection exerted by *P. brassicae* would be cancelled out by the more intense herbivory of the grazers (Gómez 1996). Considering that the plant species in our study vary in several traits (annual versus perennial, size, morphology) and the fact that they grow in different habitats, selection for egg induction may not be the same.

All plant species included in this study, are natural host plants of *P. brassicae* (Gómez 1996, Gols *et al.* 2008, Fei *et al.* 2014). *Pieris brassicae* is a voracious herbivore that can have a significant impact on plant survival and fitness of the annual *B. nigra* (Smallegange *et al.* 2007). Therefore, early recognition of infestation by herbivores, i.e. already in the egg stage, followed by a response that negatively influences development of the larvae hatching from these eggs, may reduce damage levels and potentially enhance plant fitness (Pashalidou *et al.* 2013). Other studies showed that egg induction influenced plant responses against herbivores, in different systems such as the sawfly *Diprion pini* and the pine *Pinus sylvestris* (Beyaert *et al.* 2011) and tomato (*Solanum lycopersicum*) – tomato fruitworm moth (*Helicoverpa zea*) (Kim *et al.* 2012). Thus, plant responses to eggs may have evolved as an effective protective measurement against herbivory, as they influence herbivore performance (Kim & Felton 2013).

The consequences of egg deposition for plant quality are clearly not limited to the egg stage, because egg induction affected the performance of larval stages of *P. brassicae* (this study, Pashalidou *et al.* 2013). Therefore, when studying food plant quality for insect herbivores, it is important to follow the insect herbivore until it has completed its immature development. Other parameters related to caterpillar and pupal development may also be influenced by egg induction, such as larval mortality, adult weight, and adult fecundity, but these were not included in this study. Though egg induction significantly affected larval and pupal development of *P. brassicae*, plant quality differences were most pronounced at the species level. Larval and pupal masses were lowest and development time was longest on *B. oleracea* and the two *Moricandia* species and shortest for *P. brassicae* developing on *B. nigra* and *S. arvensis.*

Our results corroborate previous studies that indicate differences in performance of *P. brassicae* when developing on different brassicaceous species. *Pieris brassicae* larval and pupal performance varied between *B. nigra* and *Brassica juncea* plants. *Pieris brassicae* butterflies that developed on *B. juncea* were the heaviest, yet their developmental time was longer (Gols *et al.* 2009). Another study showed that *P. brassicae* perform better on *B. nigra* compared to *Barbarea vulgaris* and *B. oleracea* (Sznajder & Harvey 2003). Changes in plant quality due to plant defensive chemistry can alter the structure and composition of their associated insect community (Bangert *et al.* 2006, Johnson *et al.* 2006, Poelman & Dicke 2014). The same chemical compounds that plants use to deter generalist herbivores, may be used by specialist herbivore species to recognise their food plants (Schoonhoven *et al.* 2005). Many studies have shown that natural plant species can vary in their levels of secondary plant compounds that affect food plant quality for insect herbivores. In this study, we show that in addition to the relatively larger interspecific variation in plant quality, egg induction further alters plant quality.

We show that changes in plant quality induced by egg deposition can affect herbivore performance on different plant species. We show how common and persistent this effect is across wild brassicaceous plant species and, thus, added another type of induced resistance, i.e. one that is triggered during the initial phase of plant colonization. Future work should evaluate under field conditions whether *P. brassicae* shows oviposition preference for particular Brassicaceae species in relation to egg induction or to differences in plant quality in general. It is currently unclear how strong these interactions are in natural environments and whether these traits are phylogenetically conserved within the Brassicaceae family. Moreover, molecular tools allow for the elucidation of the mechanism underlying egg induction and whether they are conserved across phylogenetically related plant species.

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Chapter 4

To be in time: egg deposition enhances plant-mediated detection of young caterpillars by parasitoids

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Animals use information from their environment while foraging for food or prey. When parasitic wasps forage for hosts, they use plant volatiles induced by herbivore activities such as feeding and oviposition. Little information is available on how wasps exploit specific plant volatiles over time, and which compounds indicate changes in host quality. In experiments investigating the role of herbivore-induced plant volatiles in wasp foraging, induction of plant response is usually achieved by placing larvae on clean plants instead of allowing the natural sequence of events: to let eggs deposited by the herbivore develop into larvae. We compared the attraction of the parasitoid *Cotesia glomerata* to volatiles emitted by black mustard (*Brassica nigra*) plants induced by eggs and successive larval stages of the Large Cabbage White butterfly (*Pieris brassicae*) to the attraction of this parasitoid to black mustard plant volatiles induced only by larval feeding in a wind tunnel setup. We show that wasps are attracted to plants infested with eggs just before and shortly after larval hatching. However, wasp preference changed at later time points towards plants induced only by larval feeding. These temporal changes in parasitoid attraction matched with changes in the chemical compositions of the blends of plant volatiles. Previous studies have shown that host quality/suitability decreases with caterpillar age and that *P. brassicae* oviposition induces plant defences that negatively affect subsequently feeding caterpillars. We investigated parasitoid performance in hosts of different ages. Wasp performance was positively correlated with preference. Moreover, parasitism success decreased with time and host stage. In conclusion, the behaviour of *Cotesia glomerata* is fine-tuned to exploit volatiles induced by eggs and early host stages that benefit parasitoid fitness.

Keywords: Host quality, HIPVs, host location, host suitability, *Cotesia glomerata*

Introduction

Plants release volatile organic compounds (VOCs) that affect the structure of plant-associated communities (Poelman *et al.* 2008, Tentelier & Fauvergue 2007). The significance of plant volatiles used by herbivores, pollinators, and carnivores (including insectivorous birds) to locate food, prey, or hosts has been thoroughly studied (Amo *et al.*2013, Dicke & Baldwin 2010). Since the late 1980s, it has been shown that many of the plant volatile compounds are induced either by feeding (i.e. herbivore-induced plant volatiles—HIPVs) or by egg-laying (i.e. oviposition induced plant volatiles—OIPVs) herbivores (Hilker & Meiners 2011, Mumm & Dicke 2010). The released volatile blends may vary with the identity, density and developmental stage of the damage-inflicting herbivore and also with plant species and their developmental stage (Clavijo McCormick *et al.* 2012). HIPVs and OIPVs can attract or repel herbivores and their natural enemies (De Moraes *et al.* 2001, de Rijk *et al.* 2013, Fatouros *et al.* 2012, Snoeren *et al.* 2007) and prime neighbouring plants or undamaged parts within the same plant (Dicke & Baldwin 2010, Frost *et al.* 2008, Kim & Felton 2013).

For parasitic wasps, host-searching success is directly linked to the production of offspring. Offspring of parasitoids rely on limited resources because they develop in or on single hosts, which grow marginally larger than the attacking parasitoid (Harvey 2005). Larvae of koinobiont endoparasitoids grow inside the host and allow the host to continue to feed and develop. Host quality and suitability depend on host growth rate, host stage, and final host size (Harvey *et al.* 1999). Therefore, the host represents a dynamic resource, and can vary in suitability and quality during the different host stages (Hilker & Meiners 2010). The ability of parasitoids to discriminate between plant volatile blends induced by different herbivores depends on the dietary specialisations of both the host and the parasitoid (Clavijo McCormick *et al.* 2012, Jervis *et al.* 2008, Mattiacci & Dicke 1995a, b, Vet & Dicke 1992). To better understand the adaptive value of parasitoid foraging decisions, studies are needed to investigate whether the preference of the parasitoid for specific volatile blends is linked to increased success in finding a host and ultimately reproductive success (Clavijo McCormick *et al.* 2012).

Parasitoids that can exploit HIPV blends, which indicate maximal host quality and suitability for their offspring, are expected to be favoured by natural selection (De Moraes *et al.* 2001, de Rijk *et al.* 2013). Plants may also activate defences upon herbivore egg deposition before the actual feeding damage starts, and this might be an effective strategy to reduce upcoming herbivory. Eggs can change the plant's physiology and subsequently the performance of feeding caterpillars (Hilker & Meiners 2011, Reymond 2013, Pashalidou *et al.* 2013). In the study reported in the present paper, we investigated whether oviposition alters plant volatile emissions before and after caterpillar feeding at different time points, and whether this affects the attraction and performance of a larval parasitoid. We linked wasp preference for HIPVs with wasp performance and assessed parasitoid attraction to volatiles induced by eggs and feeding caterpillars of different instars in a two-choice bioassay. Caterpillar developmental stage ranged from neonate until later host instars, and the caterpillars fed either on plants on which eggs had developed into larvae (eggs(+) plants) or on plants that were only exposed to larval feeding (eggs(−) plants). Chemical analysis of the headspace of these plants was also conducted. Furthermore, we assessed, at different time points, whether *P. brassicae* host stage (1-h-old neonates, L1, L2) resulted in differences in wasp performance when they developed into larvae feeding on eggs(+) plants or on eggs(−) plants.

We assessed parasitisation success and adult parasitoid biomass as proxies of *C. glomerata* fitness. Host suitability includes the behavioural ability of the wasp to parasitise different host stages and its physiological capability of growing and developing. We hypothesised that changes in parasitoid preference were based on temporal changes in HIPV signalling to enable selection of the most suitable host stage for parasitism. We used *Brassica nigra* (black mustard), which is a wild Eurasian summer annual plant native to The Netherlands (Ponzio *et al.* 2014). The gregarious larval endoparasitoid *Cotesia glomerata* is fairly specialised and attacks young caterpillars of Cabbage White butterflies (*Pieris* spp.), such as the Large Cabbage White butterfly *Pieris brassicae. Pieris brassicae* is, in turn, a specialist on brassicaceous plants (Mattiacci & Dicke 1995b). In addition, previous studies have shown that *C. glomerata* performs better in young host instars compared to later ones because larval immune defences in early host stages are limited (Brodeur *et al.* 1998, Harvey *et al.* 1999, Mattiacci & Dicke 1995b). However, *B. nigra* plants induced by eggs of *P. brassicae* negatively affect the performance of caterpillars that subsequently feed on the plants (Pashalidou *et al.*2013), so we were interested in whether this egg-induced response also affects members of the third trophic level. Moreover, *Pieris* egg deposition or feeding by caterpillars has been shown to induce volatiles in *B. nigra* plants that attract egg and/or larval parasitoids (Fatouros *et al.* 2012, 2014, Gols *et al.* 2008).

Materials & Methods

Plants and insects

Black mustard plants (*Brassica nigra* L., Brassicaceae) were grown in a greenhouse compartment (23 ± 2 °C, L16:D8, 50–70 % RH). Seeds were obtained from the Centre of Genetic Resources, Wageningen (accession number: CGN06619), The

Netherlands. Seed numbers were multiplied by exposing the plants to open pollination in a field nearby Wageningen. Four-week-old non-flowering plants with 6–8 fully expanded leaves were used for the experiments. *Pieris brassicae* L. (Lepidoptera: Pieridae) was reared on *B. oleracea* L. var. *gemmifera* cv. Cyrus plants in a climatised room (21 ± 1 °C, L16:D8, 50–70 % RH). The parasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae) was reared on *P. brassicae* caterpillars in a greenhouse compartment under similar temperature and humidity conditions as *B. nigra* plants. *Cotesia glomerata* cocoons were placed in a cage (30 × 30 × 30 cm, Bugdorm, Taiwan, China) supplied with a 6 % sucrose solution. The cage with adult wasps was positioned in a climate cabinet at 21 ± 1 °C and L16:D8. Adult wasps were allowed to mate. Only naïve females (i.e. females that had no previous experience of hosts, host products, or plants) were used in the experiments. Wasps were 3–5-days old when used in the preference and performance bioassays (see below). All individuals were used only once.

Plant treatments

Plants were treated as previously described in detail in Pashalidou *et al.* (2013). Plants exposed to *P. brassicae* eggs were denoted “eggs(+)”. Each eggs(+) plant was infested with 20 eggs by allowing butterflies to deposit eggs freely, after which any surplus of eggs was removed with a fine brush. Clean plants not exposed to insects or plants that were not exposed to egg deposition but to caterpillar feeding only (denoted “eggs(-)”) were used as controls. Five days after egg deposition (within 1 h after larval hatching), ten neonate caterpillars were transferred to the adaxial side of the same leaf on the eggs(+) plant on which they had hatched, and ten neonates were transferred to the adaxial side of a leaf of the same age on an eggs(-) plant. This procedure was repeated with new sets of eggs(+) and eggs(-) plants on which the caterpillars were allowed to develop for different time periods (1, 2, 8, 24, 48 and 72 h from hatching) before they were used as an odour source in a wind-tunnel bioassay (see below).

Preference bioassays in the wind tunnel

Host-searching behaviour of female *C. glomerata* wasps was observed in a wind-tunnel [as previously described in detail by Ponzio *et al.* (2014)]. A pair of plants were placed in the wind tunnel. First, experiments were conducted to test whether wasps discriminated between clean control plants and egg-infested plants (eggs(+)) (−24 h before hatching) and between clean control plants and eggs(-) plants infested with feeding larvae only (1, 2, 8 and 48 h from hatching). Ponzio *et al.* (2014) previously showed that wasps preferred eggs(-) plants infested with *P. brassicae* caterpillars over clean plants 24 h after hatching. Second, experiments were conducted with eggs(+) plants infested with caterpillars and tested against eggs(-) plants infested with caterpillars. The attraction of parasitoids to volatiles of eggs(+) and eggs(-) plants was tested at various time points after the larvae had hatched (1, 2, 8, 24, 48 and 72 h). Each plant was placed in the wind tunnel 30 min before the experiment to allow recovery from possible handling effects. Female wasps were tested in the wind tunnel one at a time. The behaviour of the wasps was observed until they landed on one of the two plants. This plant was recorded as the preferred plant. If they did not land on a plant within 15 min, "no response" was recorded.

The wasps that were scored as having "no response" were excluded from the statistical analysis. A total of 6–15 wasps were released for each plant-pair replicate, resulting in a minimum of five responding wasps. Ten biological replicates (plant pairs) were used per time point.

Collection of volatiles and headspace analysis

We collected plant volatiles from eggs(+) and eggs(-) plants exposed to caterpillar feeding for different durations after hatching (2, 24, 48 and 72 h) to assess whether parasitoid preference behaviour could be linked to differences in volatile profiles. In addition, we collected volatiles from plants with eggs 24 h before larval hatching (−24 h) to compare the volatile emission of eggs(+) plants with that of clean plants prior to caterpillar feeding. Air from empty jars was collected to correct for background odours. Ten replicates were sampled per plant treatment. Pots were wrapped in aluminium foil and the plants were placed in 30-l glass jars 30 min before trapping began. Compressed air was filtered through activated charcoal and volatiles were collected by drawing air out of the jars with a pump through a stainless steel cartridge containing 200 mg of Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) at a rate of 100 ml min−1 for 4 h. The aerial parts of each plant were weighed immediately after trapping. We followed the protocol for volatile trapping and compound identification described in detail in Methods S1 of the Appendices, using a Thermo Trace GC Ultra in combination with a Thermo Trace DSQ quadruple mass spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) to separate and detect plant volatiles.

Performance bioassays

To assess parasitoid performance, three neonate hosts were transferred to the adaxial side of an eggs(+) plant and three neonates to an eggs(-) plant, as described above in "Plant treatments". Larvae were parasitised by *C. glomerata* wasps as 1-h-old neonates and 1-day-old L2 on eggs(+) and eggs(-) plants, respectively. One adult female wasp was used per plant. Single females were first isolated in a glass vial with a drop of honey. Females were released on the leaf near to the caterpillars.

The females were allowed to search and parasitise three caterpillars, with one parasitisation per caterpillar; the females were then collected and the unparasitised caterpillars were removed. After parasitism, the plants were returned to the climate chamber and kept there until the larvae of *C. glomerata* egressed from the host caterpillars in the late fifth instar and pupated. During the development of the caterpillars, the plants were watered regularly and the development of the caterpillars was monitored. Parasitoid cocoons were collected in Petri dishes and subsequently placed in Bugdorm cages. The cages were kept in the same climate chamber as the plants until adult eclosion. Ten females and ten males were collected from each cage to assess dry body mass. Following adult eclosion, the parasitoids were killed in a freezer, placed in an oven at 75 °C for 24 h, and weighed individually (1 µg accuracy; Sartorius AG, Göttingen, Germany).

Parasitism success

To assess the success of the parasitism of *C. glomerata* in *P. brassicae* caterpillars of different ages, a single female wasp was released on an eggs(+) plant with 1–2 caterpillar clusters. The experimental plants had been exposed to adult butterflies until females had been observed to deposit 1–2 egg clusters on the plants. Plants were maintained in a greenhouse compartment (23 ± 2 °C, L16:D8, 70 ± 10 % RH) until the larvae hatched and developed into (a) 1-h-old neonates, (b) 1-day-old L1 or (c) 1-day-old L2. Plants were enclosed in large nets (60 x 100 cm, Bugdorm) supported by four wooden sticks that were placed in the plant pots. Wasps were released onto a leaf with caterpillars. Each wasp was allowed to search and parasitise the caterpillars of one of each of the host-age treatments for 90 min (n = 10 per treatment). After this period, the wasps were removed, and the plants with caterpillars were maintained for another 24–48 h at room temperature before the caterpillars were dissected for parasitoid egg presence. The number of eggs was counted and considered a proxy for successful parasitism.

Statistics

Differences in parasitoid preference were analysed by logistic regression. Plant treatment (eggs(+), eggs(-)) and time before and after larval hatching [(−24 h), 1, 2, 8, 24, 48, 72 h] were treated as fixed factors. In the comparison of an eggs(+) plant with an eggs(-) plant, the number of wasps that chose the eggs(+) plants out of the total number of responding wasps was used as the response variable. Each bioassay with one set of plants served as a replicate. To determine within each comparison whether there was a significant preference for one of the plant treatments offered, we tested $H0$: logit = 0. Statistical analysis was conducted in R.2.4.1 (R Development Core Team 2008). The volatile emission data, expressed as peak areas divided by the fresh mass of the plant, were subjected to principal component multivariate data analysis using the orthogonal projections to latent structures discriminant analysis (OPLS-DA) function of the SIMCA-P+ 12.0 software (Umetrics AB, Umeå, Sweden).

This projection method determines whether samples collected from the different treatment groups can be separated on the basis of quantitative and/or qualitative differences in their volatile blends.

The results of the analysis are visualised in score plots, which reveal the sample structure according to model components, and loading plots, which display the contribution of the variables to these components as well the relationships among the variables, based on the influence of each variable on the projection (VIP values) (Wold *et al.* 2001). Compounds were excluded if they were present in less than half of the samples in one or both of the treatments. We conducted pairwise OPLS-DA analyses of volatiles emitted by eggs(+) and eggs(-) plants at different time points following egg deposition: 24 h before the larvae hatched (i.e. −24 h) and at various time points after they had hatched and fed (2, 24, 48 and 72 h). The analysis was done for each time point. A pairwise (Mann–Whitney *U* test) comparison was conducted per time point for each compound on eggs(+) and eggs(-) plants (Fig. S1, Appendices).

A sign test was used to determine whether the absolute number of compounds that were emitted in substantial amounts differed from a 50:50 distribution over the two treatments for each time point (Table S1, Appendices). Performance data of the wasps were analysed using a linear mixed model fitted by restricted maximum likelihood (REML).

Plant treatment ((eggs(+), eggs(-)), host stage (1-h-old neonates and L2) and their interaction were treated as fixed factors whereas replicate was treated as a random term. Model fitting was performed using REML in R 2.4.1 (R Development Core Team 2008). Normality, independence, and homogeneity of variance were checked by inspecting the residuals after fitting the model. Parasitism was considered successful when one or more eggs were found after dissecting the hosts 24 h after parasitisation.

Parasitism success (=proportion of the hosts that were successfully parasitised) in relation to host age (1 h-old neonates, L1, and L2) was analysed using a general linear model in Genstat 16th edition SP1. Host density varied within and among treatments, and since this may influence parasitism success, it was entered as a covariate in the analysis.

Results

Plant-mediated effects of egg deposition on wasp attraction

Cotesia glomerata females significantly preferred plants with eggs (eggs(+)) over clean plants 24 h before caterpillar hatching (Table S2, Appendices). There was no preference for clean plants or plants infested with caterpillars only (eggs(-)) at 1 or 2 h of feeding, but wasps preferred plants infested with caterpillars after 8, 24 and 48 h of feeding (Table S2, Appendices). *Cotesia glomerata* females significantly preferred volatile blends emitted by eggs(+) plants infested with feeding caterpillars over volatiles from eggs(-) plants at 1, 2 and 8 h after hatching (Fig. 1, z = 2.71, $P = 0.006$ (1 h); z = 3.45, $P < 0.001$ (2 h); z = 3.42, $P < 0.001$ (8 h)). This preference was shifted when tested after 72 h of feeding, when the caterpillars had reached the L2 instar. At this time point, wasps preferred volatile blends emitted from eggs(-) plants over volatile blends from eggs(+) plants (Fig. 1, z = −2.36, $P = 0.021$ (72 h)).

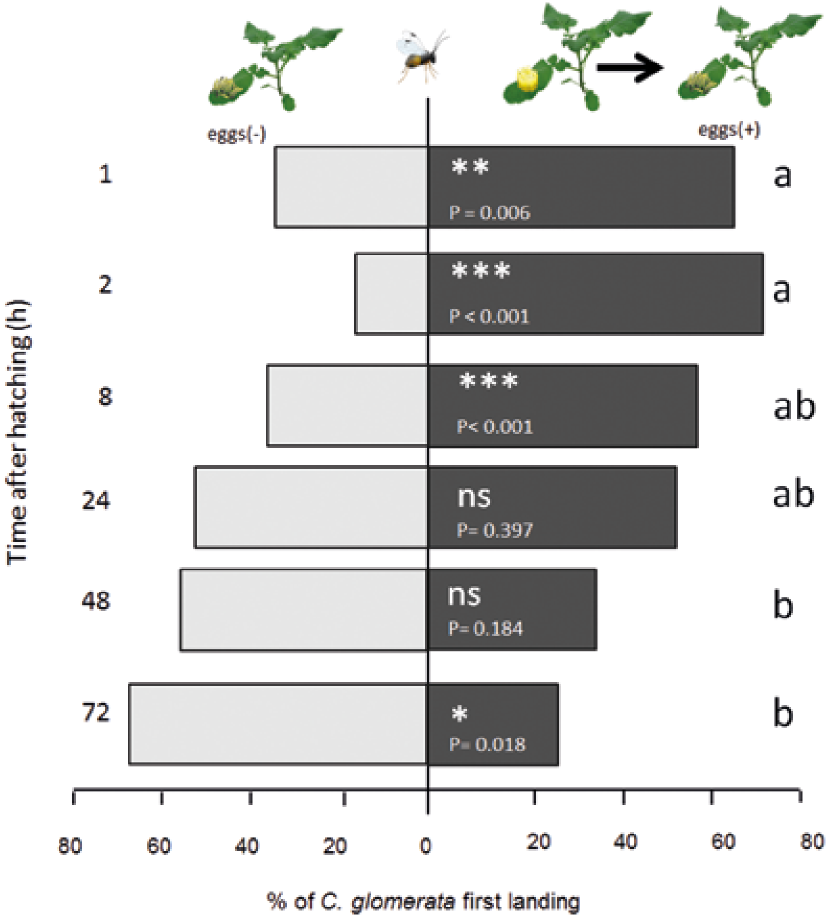

Figure 1. Percentage of first landings of *Cotesia glomerata* female wasps on caterpillar-infested eggs(+) versus caterpillar-infested eggs(-) *B. nigra* plants in a wind tunnel setup. Eggs(+) = plants were exposed to eggs and subsequently to feeding larvae, whereas eggs(−) = plants were only exposed to feeding larvae. Asterisks indicate a preference which is significantly different from a 50:50 distribution within a choice test. Significant differences between treatments ($P < 0.05$) are indicated by different letters. Ten biological replicates were conducted per time point and at least n = 6–15 wasps were tested per plant pair, *$P < 0.05$, ***$P \leq 0.001$, ns not significant, (GLM).

Plant volatile analysis

In total, 39 volatile compounds were detected across all experimental treatments [caterpillar-infested eggs(-) and caterpillar-infested eggs(+) plants at 2, 24, 48 and 72 h after caterpillar hatching] and were present in more than 50 % of the replicates (Table S1, Appendices). No qualitative differences were found, but there were quantitative differences in the compositions of the blends depending on egg treatment and duration of caterpillar infestation (Fig. 2, Fig. S1, Table S1, Appendices). Pairwise comparisons by OPLS-DA for each time point resulted in models with two significant principal components at two time points, i.e. 2 h (Fig. 2a) and 48 h (Fig. 2b) after hatching of larvae, and with one significant principal component at 72 h after egg hatching (Fig. 2c). A group of 12 compounds with VIP values ≥ 1.0 contributed the most to the differentiation between the blends at 2 h after the larvae hatched (Fig. 2a). The sesquiterpene 7-epi-α-cedrene was released in significantly higher amounts by eggs(+) plants than by eggs(-) plants (z= -0.092, *P* = 0.02, Fig. S1, Table S1, Appendices). Two compounds were almost significantly suppressed, namely the sesquiterpenes longicyclene and β-gurjunene (z = -1.984, *P* = 0.06 and z = -1.886, *P* = 0.06, Fig. S1, Table S1, Appendices). A group of 12 compounds with VIP values of ≥1.0 contributed the most to the differentiation between the blends at 48 h after egg hatching (Fig. 2b). The average total amount of volatiles from caterpillar-infested eggs(-) plants was significantly higher than that from caterpillar-infested eggs(+) plants (z = −2.57, *P* = 0.01, Fig. S1, Table S1, Appendices). A group of 12 compounds with VIP values of ≥1.0 contributed the most to the differentiation between the blends at 72 h after egg hatching (Fig. 2c). The green-leaf volatile (*Z*)-3-hexen-1-ol was emitted in significantly larger amounts from caterpillar-infested eggs(+) plants than from caterpillar infested eggs(−) plants (z = −2.57, *P* = 0.01, Fig. S1, Table S1, Appendices).

Parasitoid performance

Cotesia glomerata wasps generally grew larger when their mothers had parasitised 1 h-old neonates rather than L2 caterpillars of *P. brassicae*, irrespective of whether the caterpillars developed on eggs(+) plants or eggs(-) plants. The observed differences in adult dry mass were similar for male and female wasps (females: $F_{1,285}$ = 4.91*, P* = 0.03; Fig. 3a, males: $F_{1,285}$ = 20.74*, P* < 0.001; Fig. 3b). However, when we compared the development within caterpillars of the same age (1-h-old neonates or L2), wasps were observed to grow larger when the hosts fed on eggs(-) plants rather than on eggs(+) plants (females: $F_{1,285}$ = 40.6*, P* < 0.001; Fig. 3a, males: $F_{1,285}$ = 110.87*, P* < 0.001; Fig. 3b). The parasitism success of *C. glomerata* was significantly lower on a patch with L2 caterpillar hosts than on a patch with either 1-h-old neonates or L1 (host stage, $F_{2,26}$ = 4.72, *P* = 0.02; Fig. 4). Parasitism success was negatively correlated with host density (host density $F_{1,26}$ = 10.9, *P* = 0.003; Fig. 4).

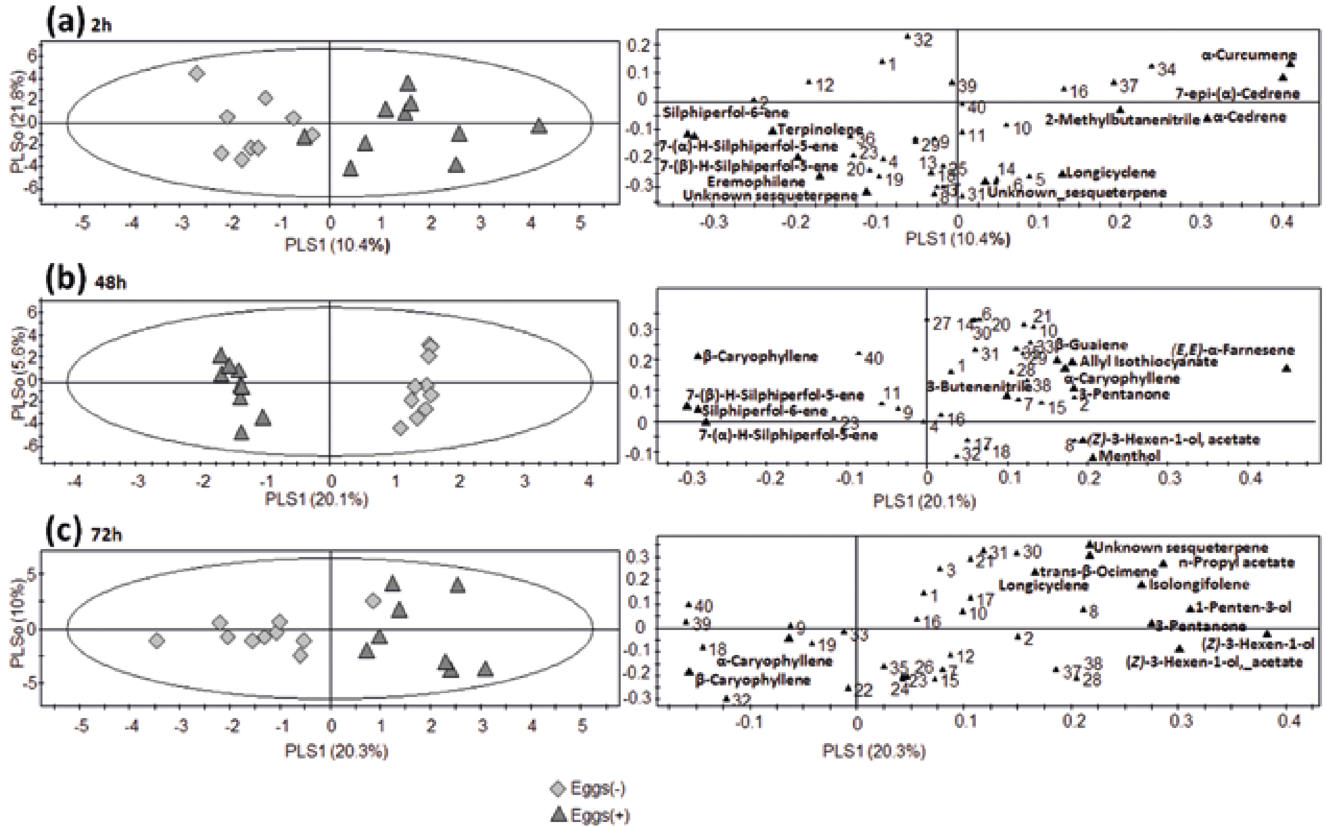

Figure 2. Orthogonal projections to latent structures discriminant analysis (OPLS-DA) of volatile compounds collected from caterpillar-infested *B. nigra* plants previously exposed to egg deposition(eggs(+), *triangles*) or not (eggs(-), *diamonds*). An OPLS-DA was done on the peak area of log-transformed data from headspace volatiles. Score plots and loading plots of samples (a) 2 h, (b) 48 h and (c) 72 h after hatching, according to the first two principal components and the Hotelling *T*2 ellipse confining the confidence region (95 %), with the most influential volatiles (VIP ≥ 1.0) labelled in the loading plot.

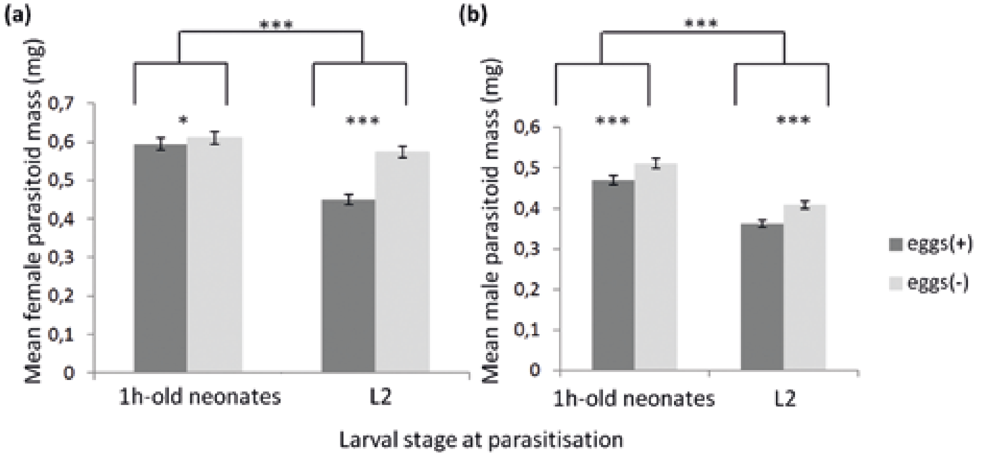

Figure 3. *Cotesia glomerata* adult dry weight. Mean dry weights (±SEM) of adult F1 (a) female and (b) male wasps that developed in *P. brassicae* caterpillars which fed on either eggs(+) or eggs(-) *B. nigra* plants and resulted from parasitisation of 1-h-old neonates or L2. Asterisks indicate significant differences between and within treatments. $*P < 0.05$, $***P \leq 0.001$, (LMM).

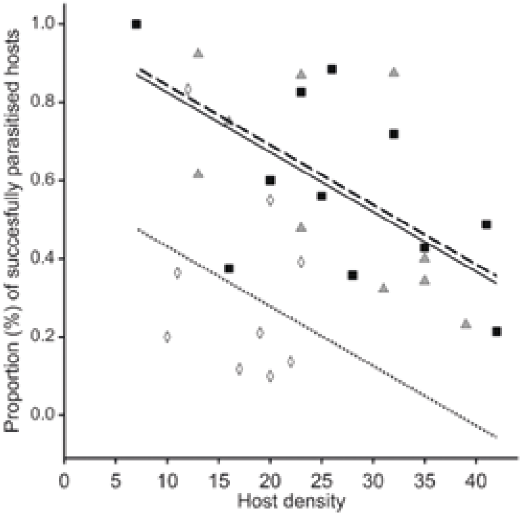

Figure 4. *Cotesia glomerata* successful parasitism (proportion of hosts parasitised) of different host developmental stages in relation to host density. (1 h neonates: *filled squares*, *dashed line*; L1: *filled triangles*, *solid line*; L2: *open diamonds*, *dotted line*), (GLM).

Discussion

Our data show that *P. brassicae* egg deposition alters subsequent HIPV emission in response to caterpillar feeding and parasitoid attraction over time. During the first hours of herbivory, female *Cotesia glomerata* parasitoids preferred HIPV blends emitted by eggs(+) plants that were infested with eggs and fed upon by caterpillars over HIPV blends emitted by plants that were only exposed to caterpillar feeding. This preference for HIPVs from eggs(+) plants changed over time to a preference for eggs(-) plants, which correlates with a change in host quality after egg hatching.

Parasitism success decreases with time: the older the larvae tested, the lower the parasitism rate in larvae feeding on eggs(+) plants. As host quality and success rate decrease with host age over the first few days after egg hatching, it is expected that natural selection will favour females that locate very young host larvae, even those that are yet to hatch from their eggs (Fatouros *et al.* 2012).

Our results agree with the prediction of Vet & Dicke (1992) that detectable and reliable information provided by herbivore-infested plants is used by specialised parasitoids, promoting searching efficiency, and thus Darwinian fitness. The host-searching behaviour of *Cotesia glomerata* has been intensively studied: the wasps can discriminate between caterpillar-infested plants and clean plants in both wild and cultivated brassicaceous plants (Gols *et al.* 2008). *Cotesia glomerata*, however, did not discriminate between HIPVs induced by different larval stages of *P. brassicae* (L1 vs L5) in Brussels sprouts (Mattiacci & Dicke 1995a, b).

In our study of *B. nigra*, the volatile blend changed over time with caterpillar development and could be a reliable signal for *C. glomerata*, indicating the location of high-quality hosts. In terms of parasitoid performance, host quality is lower at a later developmental stage on *B. nigra* plants that were previously infested with eggs compared to egg-free plants (Pashalidou *et al.* 2013). Effects of plant defences against herbivores cascading up to parasitoids have been reported for different wild *B. oleracea* populations, the herbivores *P. rapae* and the cabbage moth *Mamestra brassicae*, and their endoparasitoids *C. rubecula* and *Microplitis mediator*, respectively (Gols *et al.* 2008). In addition, in *Plantago lanceolata*, the development of the two parasitoids *Melitaea cinxia* and *Hyposoter horticola* paralleled the performance of their herbivorous hosts (Harvey *et al.* 2005). Secondly, the immune responses of caterpillars against their enemies increase as the caterpillars develop to later instars (Brodeur *et al.* 1998, Harvey *et al.* 1999, Mattiacci & Dicke 1995b). Moreover, in nature, *P. brassicae* larvae in particular move from the plants on which they hatched until the final larval instars (Smallegange *et al.* 2007).

Therefore, testing the effects of caterpillars has limited relevance from an ecological point of view. To conclude, it is important for the wasps to find their *P. brassicae* hosts as soon as possible, since the wasps suffer from (a) egg-induced plant defences and (b) the immune defences of the caterpillar as they grow. Volatile analysis reveals that the volatile blends changed over time. The wasps may perceive these changes, and this may affect their host-searching behaviour. Insects potentially detect differences between volatile blends due to (a) the absence or presence of specific compounds in the blend, so-called "species-specific odour recognition," (b) changes in the ratios of compounds within the blends, so-called "ratio-specific odour recognition," or (c) perception of the entire blend, so-called "whole-blend odour recognition" (Clavijo McCormick *et al.* 2012). In this study, no significant differences in individual compound emission were found, apart from two compounds at two time points.

However, our study and a previous study by Fatouros *et al.* (2012) support ratio-specific odour recognition because the same compounds were detected in the headspace of caterpillar-infested eggs(+) and eggs(-) plants at each time point, whereas their ratios changed with time. Thus, our study underlines the importance of the quantitative composition of the HIPVs for host-searching parasitoids. More experiments, as described for example by De Boer *et al.* (2004), are required to establish if certain individual compounds in the HIPV blend play more important roles in wasp attraction than others. A recent exciting study shows that birds (great tits) can discriminate between HIPVs emitted by trees infested with caterpillars and clean trees (Amo *et al.* 2013). However, not only carnivores but also other community members, including “friends” (e.g. pollinators) and “foes” (e.g. herbivores), respond to HIPVs, so the benefit to the plant should be seen in a community context (Dicke & Baldwin 2010, Kessler & Heil 2011). Here, we demonstrated that the odour-based preference of the wasps varied in response to changes in the HIPV blends emitted at different times after hatching. The wasps’ preference correlates with host suitability in terms of parasitism success and parasitoid development. Indeed, herbivore- induced changes in plant phenotype can alter insect communities over different time scales: from minutes until years (Stam *et al.* 2014). The temporal dynamics of HIPVs have been studied in many systems, including cotton (Loughrin *et al.* 1994), maize (Turlings *et al.* 1998), maple (Ping *et al.* 2001) and apple fruits (Hern & Dorn 2001). However, there are few studies that have linked the temporal dynamics of plant volatile emission with the preferences and performance benefits for parasitoids. Scascighini *et al.* (2005) reported that *C. glomerata* was attracted to the HIPVs of Brussels sprout plants infested with 50 L2 caterpillars 1–14 h after feeding rather than those of clean plants. Moreover, the temporal dynamics of HIPVs can influence the interactions between the plant (*Brassica juncea*), its herbivores (*Spodoptera litura*, *Plutella xylostella*) and their parasitoids (*Cotesia marginiventris*, *Cotesia vestalis*), and elicit different preferences for herbivores and parasitoids (Mathur *et al.* 2013).

The generalist *S. litura* preferred undamaged plants, whereas its parasitoid preferred 48-h-damaged plants. The specialist *P. xylostella* and its parasitoid, however, favoured 72-h-damaged plants over undamaged plants or plants damaged for 24 and 48 h. *Cotesia karyai* discriminated between HIPVs from maize plants induced by different ontogenic stages of *Pseudaletia separate* (Takabayashi *et al.* 1995). In maize, however, when *Microplitis rufiventris* was investigated for its response to plant volatiles induced by different larval stages of *Spodoptera littoralis*, neither volatile emission nor parasitoid behaviour varied (Gouinguené *et al.* 2003). All in all, the composition of the HIPV blend changes with time, and the responses of various insects to these volatiles may vary accordingly (Mathur *et al.* 2013, Scascighini *et al.* 2005, Takabayashi *et al.* 1995). The time window for *C. glomerata* to find and successfully parasitise its host is narrow. Interestingly, *P. brassicae* caterpillars feeding on *B. nigra* plants during the reproductive stage have evolved a strategy to escape from *C. glomerata*: they move up to the flowers when they reach the L2 stage (Lucas-Barbosa *et al.* 2014).

Therefore, exploiting HIPVs that indicate the earliest larval stage may be highly adaptive. In a field study, parasitizing *C. glomerata* wasps were indeed observed on just-hatching egg clutches on *B. nigra* (chapter 5 of this thesis). In the present study, headspace analysis of *B. nigra* volatiles revealed clear differences between the HIPVs emitted by plants on which larvae hatched from eggs and the HIPVs emitted from plants that were only exposed to larvae. In maize (*Zea mais* L.), oviposition by *S. frugiperda* suppressed HIPV emission when it was followed by a treatment mimicking larval feeding (Peñaflor *et al.* 2011). The emission of herbivore-induced monoterpenes and homoterpenes was significantly suppressed in plants that were previously exposed to eggs. Because terpenes are likely involved in parasitoid attraction, suppressing their emission may be a strategy employed by the herbivores to circumvent parasitism or predation (Peñaflor *et al.* 2011). However, the attraction of parasitoids remains to be tested for this system (*Z. mais* and *S. frugiperda*).

Similarities are emerging, though, between the behaviour of specialist egg and larval parasitoids regarding the exploitation of volatile blends that indicate higher host quality and suitability. Firstly, (egg-) larval parasitoids can be attracted to OIPVs of host plants just before hatching, when hosts become available for parasitism by larval parasitoids.

This has been shown in different study systems, such as African grass (*Brachiaria brizantha*) with stemborer moth eggs (Bruce *et al.* 2010), spiked speedwell (*Veronica spicata*) with nymphalid butterfly eggs (Pinto-Zevallos *et al.* 2013), maize (*Zea mays*) with eggs of stemborer moths (Tamiru *et al.* 2011) and *B. nigra* with *P. brassicae* eggs (Fatouros *et al.* 2012). In those studies, it is suggested that OIPVs may be a reliable signal for larval parasitoids to arrive in host patches in time to locate their forthcoming hosts (Bruce *et al.* 2010, Fatouros *et al.* 2012, Peñaflor *et al.* 2011).

Our study shows that finding young caterpillars may have fitness benefits, as it is positively correlated with fitness proxies. Because parasitisation success decreases with time, wasps likely evolved a fine-tuned olfactory system to use these changes in the volatile blends to locate the most suitable host stage for parasitism. Secondly, egg parasitoids also use OIPVs to locate eggs that are more suitable for parasitism. The behaviour of the egg parasitoid *Closterocerus* (former *Chrysonotomyia*) *ruforum* in response to OIPVs emitted by Scots pine twigs infested with pine sawfly (*Diprion* *prini*) eggs was compared to its behaviour in response to volatiles from clean twigs at different time points. The egg parasitoids were fine-tuned to locate their hosts by only responding to OIPVs 72 h after oviposition, when eggs were more suitable for parasitism (Kopke *et al.* 2008, 2010). *Trichogramma brassicae* females, egg parasitoids of *P. brassicae* eggs, were only attracted by close-range cues of egg-infested leaves from Brussels sprout plants when the eggs were 3 days old, at which time they were most suitable for parasitism (Fatouros *et al.* 2005). Egg and larval parasitoids rely on compositional differences between HIPV blends to increase their chances of finding a suitable host plant or prey (Vet and Dicke 1992, Dicke & Baldwin 2010).

Taken together, the emerging pattern may be that egg and larval parasitoids behave in a similar way, exploiting plant cues, which indicate the most suitable host stage over time for their own fitness benefit. We have shown that temporal dynamics and egg deposition affect plant volatile emission, providing information on host stage to a larval parasitoid. Chemical analysis of the plant volatiles showed differences between blends emitted by plants on which eggs were allowed to develop to larvae and those emitted by plants that were only exposed to larvae. Jointly, our data demonstrate that differential behavioural responses to HIPV blends are positively correlated with host quality in terms of parasitism success and parasitoid performance.

This supports the notion that natural selection acts strongly on the host-searching efficiency of parasitic wasps, as this ability is correlated with offspring production. The results of this study suggest that volatile mediated host-searching behaviour of parasitoids acts in concert with plant responses to early herbivory and results in fitness benefits for the parasitoids.

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Chapter 5

Early herbivore alert matters: plant-mediated effects of egg deposition on higher trophic levels benefit plant fitness

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Induction of plant defences after the commencement of herbivore attack can reduce the metabolic cost of maintaining defences when herbivores are absent. Plants may suffer considerable damage during the time needed to mount these defences against a new attacker. Early herbivore alert is a response to cues reliably indicating future herbivory, such as egg deposition, and may be crucial for the fitness benefit of induced defences. In a field experiment, we showed that egg deposition by the specialist butterfly, *Pieris brassicae*, on the annual plant black mustard (*Brassica nigra*) induced a plant response, which negatively affected feeding caterpillars and cascaded up to the third (larval parasitoids) and fourth trophic level (hyperparasitoids) by changing insect parasitisation rate and performance. Egg deposition induced direct and indirect defences that positively affected plant fitness. Plant responses to eggs that precede herbivore attack may play an important role in the evolution of induced plant defences.

Keywords*:* Brassicaceae, egg deposition, subsequent herbivory, performance, specialist herbivore, variation

*Published in a slightly modified form.

Introduction

The use of early warning cues by organisms as a defensive strategy against predators is common in many aquatic and terrestrial ecosystems. Early signs of predation risk that induce a behavioural change in the prey to avoid predators have been found for animals ranging from arthropods to vertebrates (Lazarus 1979, Burger 1984, Wisenden *et al.* 1995, Uetz *et al.* 2002, Lima & Dill 1990, Dicke & Grostal 2001). Plants form the basis of many food webs and are, therefore, exposed to attack by a diverse community of herbivores. To cope with the diversity and unpredictability of attack, plants have evolved fine-tuned defensive traits that are often plastically induced upon herbivory. These traits known as direct and indirect induced defences can have a direct negative impact on growth performance of herbivores and/or lead to the recruitment of higher trophic level organisms (Dicke & Hilker 2003, Poelman & Dicke 2014). Recent theory suggests that plasticity of plant defences will be favoured if plants use reliable information about their environments to respond appropriately to herbivores (Karban *et al.* 1999). Karban (2011) for example reported many species of plants that use a prior herbivore attack as a reliable cue of increased risk of future herbivory, which suggests that early warning cues are a critical phase in induced defences. Apart from herbivory, volatile cues emitted from herbivore-induced plants can be used as reliable information from neighbouring plants to respond upon herbivore attack (Erb *et al.* 2015, Heil & Ton 2008).

Plants may recognise and use eggs as an early warning cue of future herbivore attack (Hilker & Fatouros 2015). Changes in plant quality and chemistry induced by egg deposition are emerging as important mediators of plant responses against future herbivory (Hilker & Meiners 2011, Hilker & Fatouros 2015, Reymond 2013, Kim & Felton 2013). Such plant-mediated effects of egg deposition can reduce the performance of the emerging herbivore, or attract its natural enemies (Beyaert *et al.* 2011, Pashalidou *et al.* 2013, 2015a). Up to now, such responses have been shown under laboratory conditions for Large Cabbage White (*Pieris brassicae*) caterpillars when feeding on different brassicaceous plants, including *Brassica nigra* (Pashalidou *et al.* 2013, 2015b) and *Arabidopsis thaliana* (Geiselhardt *et al.* 2013) and for pine sawfly (*Diprion pini*) larvae feeding on pine trees (*Pinus sylvestris*) (Beyaert *et al.* 2011).

Recognition of eggs by plants can be important because plants can use this recognition to anticipate future herbivory, and direct the investment in resources from reproduction to fight against attackers. Although proving the effectiveness of plant defences under natural conditions is challenging, some studies have demonstrated that both direct and indirect defences can reduce the negative impact of herbivores and therefore be favoured by natural selection (Agrawal 1998, Schuman *et al.* 2012, Gols *et al.* 2015). It is particularly relevant to understand at food chain level how egg deposition and caterpillar feeding trigger direct and indirect plant defences, how egg deposition affects all members of the trophic chain (herbivores, carnivores and second order carnivores), and what the ultimate benefits of such plant responses to egg deposition are for plant fitness in the field.

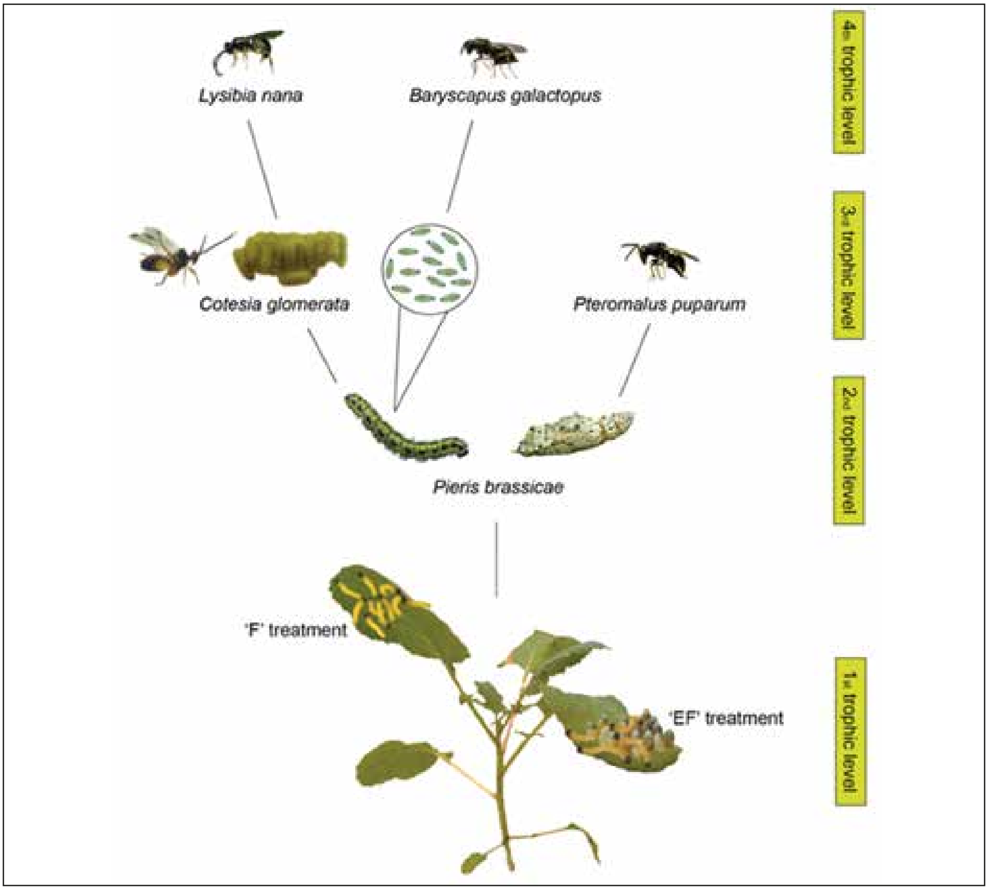

Figure 1. Trophic web on *Brassica nigra* plants studied in the field. Primary parasitoids of the third trophic level attack the caterpillars (i.e. the gregarious endoparasitoid *Cotesia glomerata*) and pupae (i.e. the gregarious endoparasitoid *Pteromalus puparum*) of the Large Cabbage White butterfly *Pieris brassicae* of the second trophic level. The larvae of the primary parasitoid *C. glomerata* inside the herbivore host are attacked by the hyperparasitoid *Baryscapus galactopus* and *C. glomerata* cocoons by *Lysibia nana*, both wasps belonging to the fourth trophic level. The effects of the two different treatments EF (i.e. exposing plants to *P. brassicae* Egg deposition and subsequent larval Feeding) and F (plants exposed to larval Feeding only) were tested on the performances and the parasitisation rates of insects at the second, third and fourth trophic levels.

In the present study, we used an experimental set-up in the field, to study the consequences of egg deposition by the specialist butterfly *P. brassicae* for direct and indirect defences against its voracious caterpillars on the annual plant *B. nigra*.

Across two consecutive field seasons, we tested the hypothesis that the plant uses egg deposition as information to anticipate upcoming herbivory by feeding caterpillars.

We explored the community-wide consequences of such plant-insect egg interactions at different trophic levels and consequences for the plants' reproductive output (Fig. 1). More specifically, we tested whether egg deposition induces plant defences that affect (a) the herbivores hatching from these eggs by reducing their performance (direct defence), and that of their natural enemies, i.e. parasitoids, (b) the attraction of parasitoids at the third trophic level (indirect defence) and natural enemies of parasitoids at the fourth trophic level, and (c) ultimately plant fitness. Furthermore, we explored the mechanistic basis of such interaction by testing whether the plant-mediated effects of egg deposition can be mimicked by using the chemical elicitor benzyl cyanide (BC). Previously, BC has been shown to be present in the egg-associated secretion released by *P. brassicae* butterflies during egg deposition and has been found to trigger an indirect plant defence against the eggs (Fatouros *et al.* 2008).

Materials & Methods

Plants and insects

In this study, we used the black mustard plant (*Brassica nigra* L., Brassicaceae), a short-lived annual, native in The Netherlands. Seeds were obtained from a natural population in The Netherlands and when needed propagated by open pollination in the field. Plants were grown in pots filled with potting soil and transplanted to the field when they were 2-3 weeks old (6-7 leaves on average). Mated adult females of the Large Cabbage White butterfly, *Pieris brassicae* L. (Lepidoptera: Pieridae), were obtained from the rearing at the Laboratory of Entomology, Wageningen University that is maintained on Brussels sprouts (*B. oleracea*, var. *gemmifera* L. cv. Cyrus) in a climatised room (21 ± 1 ˚C, 50–70 % relative humidity, L16:D8).

Experimental design

The experiment was carried out in two subsequent years (2012 and 2013) in two different fields of Unifarm, the experimental farm of Wageningen University, The Netherlands. In 2012, the field was located southwest of Wageningen (51.9515N, 5.6363E), and in 2013, the field was north of Wageningen (51.9894N, 5.6663E). In both years, the experimental site was similarly arranged, containing 15 plots each with six four-week-old *B. nigra* plants planted in a 2x3-grid design with a spacing of 1.4 m between the plants and 3 m spacing between the plots. A mixture of *Lolium* and *Poa* grasses was sown in between the plots. Two different experiments were performed by exposing plants to (a) *Pieris brassicae* egg deposition or to (b) benzyl cyanide (BC) as a chemical elicitor involved in egg-induced plant responses. Within each plot, each of the six plants was assigned to one of the following treatments: one plant infested with eggs from which caterpillars hatched (EF treatment: Egg - Feeding caterpillars), one plant infested with caterpillars only (F treatment: Feeding caterpillars). One plant treated with butterfly anti-aphrodisiac benzyl cyanide tested as chemical elicitor and caterpillars (BCF treatment: Benzyl Cyanide and Feeding caterpillars treated plants). One plant treated with 70% ethanol solution (the solvent of BC as control) and caterpillars (EtOHF treatment: Ethanol and Feeding caterpillars treated plants), two plants were left untreated (C treatment: Control plants that were left untreated).

To obtain egg-infested plants, one plant per plot was directly infested in the field by covering it with a fine net and by introducing one mated *P. brassicae* female under the net. Each female was observed up to 1 h until it deposited a clutch of approximately 40 eggs on a single leaf, and then both the butterfly and the net were removed. Over the subsequent days, plants were checked daily until caterpillars hatched. After hatching, ten caterpillars were transferred to the adaxial side of the same leaf of the same plant (treatment EF), and ten caterpillars were transferred to a different plant in the same plot that had not been exposed to oviposition (treatment F).

The remaining 20 caterpillars from the EF treatment were used to test the effect of BC. Ten caterpillars were transferred to a plant on which we had applied 100 µl of a solution of 0.01 ng BC/µl (treatment BCF: the concentration was the same as applied in previous studies by Fatouros *et al.* 2008). Ten caterpillars were transferred to a plant where ethanol had been applied (treatment EtOHF). In both cases, the solution was applied on the outer edge of the abaxial side of the fourth leaf from the top. To obtain egg depositions, five plants per day were covered with the nets and exposed to gravid butterflies. During the period when infestations were performed, BC was applied only once (in 2012) and every day during the egg phase of the EF plants (in 2013) to stronger mimic egg deposition. Within the same plot, plants were randomly assigned to each of the five treatments (EF, F, BCF, EtOHF, or C).

Field observations

In both years, all plants were exposed to the natural insect community from May until September. At 3 and 7 days after egg eclosion, caterpillar mass was assessed as a measure of fitness performance on a microbalance (accuracy= 1 µg; Sartorius AG, Göttingen, Germany). After day 7, plants were inspected every three days and parasitised caterpillars were recorded based on the presence of a parasitoid cocoon. Cocoons were collected and reared in the laboratory until adult parasitoids or hyperparasitoids emerged. *Pieris brassicae* pupae were also collected, weighed (second measure of performance) and reared until either the butterfly or pupal parasitoids emerged. Following adult eclosion the parasitoids were placed in a freezer, and thereafter in an oven at 75 °C for 24 h and adult dry weight was measured using a microbalance (accuracy= 1 µg; Sartorius AG, Göttingen, Germany) as a measure of parasitoid fitness performance. Parasitism rates by primary larval parasitoids were estimated based on the total number of caterpillars obtained from each plant by the end of the field experiment (i.e. sum of parasitised caterpillars and those that reached the pupal stage). Parasitism by pupal parasitoids and hyperparasitoids was estimated from the collected *P. brassicae* pupae, or primary parasitoid cocoons, respectively. In 2012, the effect of the experimental treatments on plant fitness was assessed by quantifying number, weight and germination rate of seeds collected at the end of August-until the beginning of September from all individual plants, including control plants (C) that had not been exposed to experimental herbivory by *P. brassicae*. Total seed number was estimated for each plant as total seed weight divided by the weight of one hundred seeds. Seeds were counted with a CONTADOR "E," Pfeuffer build 1992. The counting speed was set to 55% of maximum and the counting was set to 100 seeds. The amount of seeds counted were weighted using a scale (All scales® Europe, Model: NL-320-C-M (Max.: 320g, min: 10 mg, accuracy: 1 mg)). Germination rates were estimated on a subset of 100 randomly selected seeds per plant. Seeds were placed in a Petri dish with filter paper saturated in distilled water and the proportion of seeds successfully germinating was assessed after four days (18 ± 4 ˚C, 60-80% r.h., L16:D8).

Statistics

Differences in caterpillar, pupal, parasitoid, and hyperparasitoid weight were analysed with a mixed linear model fitted with restricted maximum likelihood (REML) with the lmer function in the lme4 package in R. Fixed effects were tested based on approximated F-tests. Data from each year were analysed separately due to large between-year variations in the community of parasitoids obtained. Biomass was treated as the observed value; treatments were entered as fixed categorical effects (i.e. first model: (EF) and (F), and second model: (BCF) and (EtOHF)). Plants nested within a plot were treated as a random effect to account for the non-independence of individual plants measured in the same plot. Differences in parasitism rates were analysed with ANOVA. Differences in seed number, seed weight and germination rate were modelled with generalised linear effects models with treatments as categorical fixed factors.

In the models, for seed weight a Gaussian error distribution was assumed, whereas for seed number and germination rate a Poisson error distribution was used. To account for over-dispersion in Poisson models the "quasipoisson" family distribution in R was used. A linear regression was also computed to assess the relationship between the three different traits related to plant reproductive output. The significance of the P values was corrected for multiple comparisons with the Bonferroni adjustment. In both generalised and mixed generalised models, assumptions on normality, homogeneity, and independence were checked by inspection of the residuals. All analyses were conducted using the R software version 2.13.1 (R Development Core Team 2008).

Results

Effects on herbivore and parasitoid performance

Pieris brassicae caterpillars had a lower biomass when fed on plants that had been previously exposed to egg deposition (EF) than when fed on plants not exposed to egg deposition (F). Differences were significant in both years, and at several developmental stages including three-days-old caterpillars (2012: $F_{1,15}$ = 56.03, $P < 0.001$; 2013: $F_{1,15}$ = 5.12, $P = 0.025$; Fig. 2a), seven-days-old caterpillars (2012: $F_{1,15}$ = 67.83, $P < 0.001$; 2013: $F_{1,15}$ = 7.41, $P = 0.007$; Fig. 2a), and pupae (2012: $F_{1,15}$ = 24.07, $P < 0.001$; for 2013: $F_{1,15}$ = 8.65, $P = 0.006$; Fig. 2a). For both years, the biomass was significantly lower among larval parasitoids *Cotesia glomerata* that developed into adults in hosts fed on EF plants than in hosts fed on F plants (2012: $F_{1,15}$ = 21.83, $P < 0.001$; 2013: $F_{1,15}$ = 21.66, $P < 0.001$; Fig. 2b), but differences were not significant for the pupal parasitoid *Pteromalus puparum* (2013: $F_{1,15}$ = 0.17, $P = 0.676$; Fig. 2b). Two hyperparasitoids were more abundant in 2013 compared with 2012: the gregarious *Baryscapus galactopus* that attacks the larvae of its primary host *C. glomerata* and *Lysibia nana* that attacks the pupae of *C. glomerata.* Similar to their parasitoid host, the hyperparasitoids had a lower biomass when they developed in hosts on EF plants previously exposed to oviposition compared with F plants exposed only to caterpillars (*B. galactopus*: $F_{1,15}$ = 5.05, $P = 0.026$; *Lysibia nana*: $F_{1,15}$ = 8.20, $P = 0.005$; Fig. 2c).

Treatment with benzyl cyanide (BC) significantly reduced the biomass of *P. brassicae* caterpillars after three (2012: $F_{1,15}$ = 18.11, $P < 0.001$; 2013: $F_{1,15}$ = 8.50, $P < 0.001$; Fig. S1, Appendices) and seven days of feeding (2012: $F_{1,15}$ = 12.70, $P < 0.001$; 2013: $F_{1,15}$ = 18.72, $P < 0.001$; Fig. S1, Appendices) on BCF plants when compared with EtOHF plants treated with ethanol only.

However, the mass of *P. brassicae* pupae, was not significantly affected by BC treatment (2012: $F_{1,15}$ = 0.44, $P = 0.506$; 2013: $F_{1,15}$ =0.14, $P = 0.710$; Fig. S1, Appendices). BC treatment affected neither the biomass of parasitoids of the third trophic level, i.e. *C. glomerata* (2012: $F_{1,15}$ = 0.58, $P = 0.448$; 2013: $F_{1,15}$ = 1.95, $P = 0.174$; Fig. S1, Appendices) and *P. puparum* (2013: $F_{1, 15}$ = 1.09, $P = 0.299$; Fig. S1, Appendices), nor the biomass of hyperparasitoids, i.e. *B. galactopus* (2013: $F_{1, 15}$ = 1.17, $P = 0.204$; Fig. S1, Appendices) and *L. nana* (2013: $F_{1, 15}$ = 1.46, $P = 0.245$; Fig. S1, Appendices).

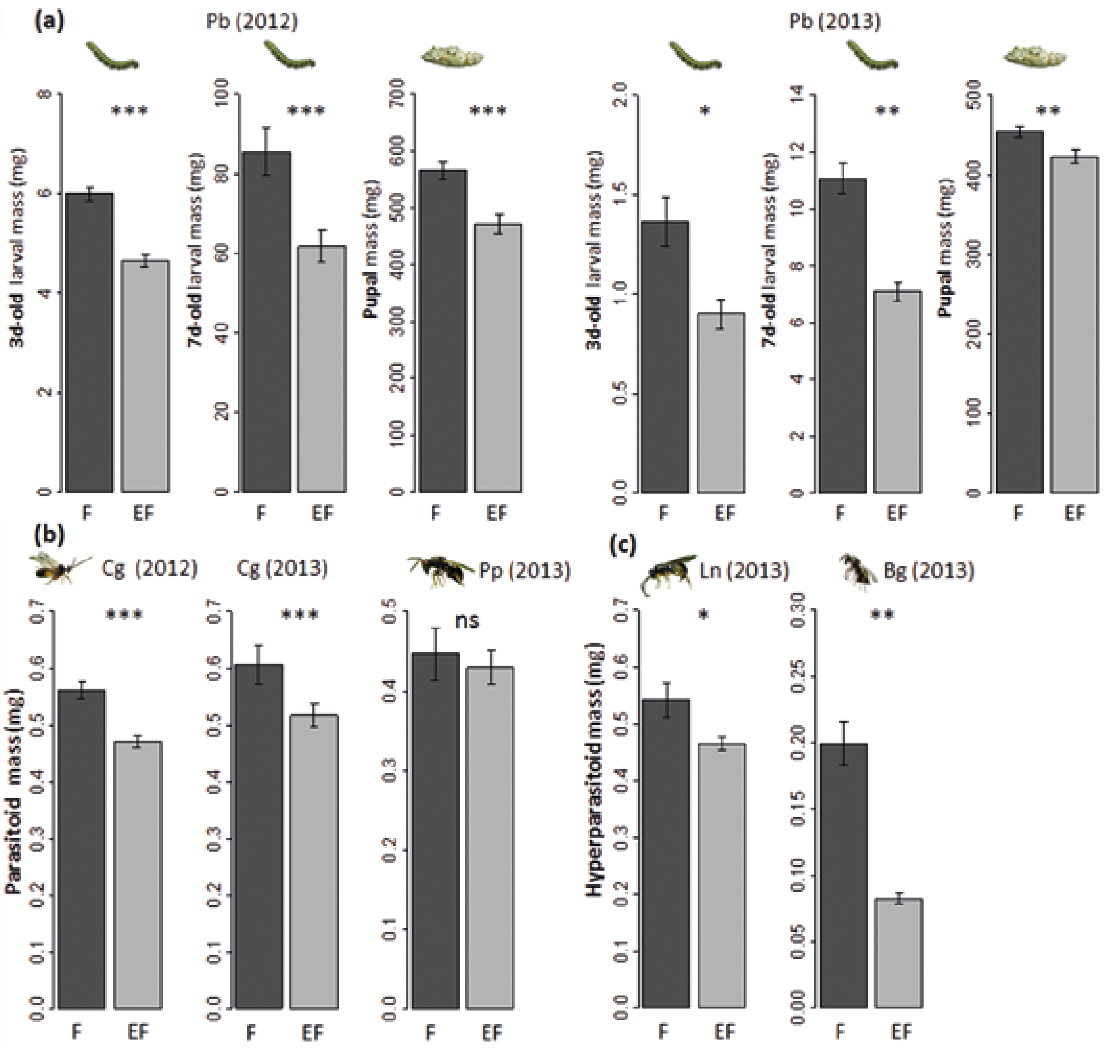

Figure 2.

Plant-mediated effects of *Pieris brassicae* oviposition on caterpillar and pupal performance under field conditions for two sequential years (2012, 2013). Bars indicate

(a) biomass (mean ± SE) of *P. brassicae* (Pb) caterpillars and pupae that developed on *B. nigra* plants: previously infested with eggs (EF), or on plants infested only with caterpillars (F),

(b) *C. glomerata* (Cg) larval parasitoids and *P. puparum* (Pp) pupal parasitoids that developed in caterpillars or pupae in *B. nigra* plants: either on EF or F plants and

(c) *L. nana* (Ln) and *B. galactopus* (Bg) hyperparasitoids that developed in *C. glomerata* on EF and F plants. Asterisks indicate significant differences in mass between the different treatments. Number of plants, n = 60. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$, ns: not significant, (LMM).

Effects on parasitism rates

In the field experiments of 2012 and 2013, we obtained parasitoids from *P. brassicae* caterpillars and pupae and hyperparasitoids from the broods of *C. glomerata* (Table 1). In both 2012 and 2013, egg parasitoids were not found to parasitise the *P. brassicae* eggs. During both years parasitism rates of *C. glomerata* were significantly higher in caterpillars that developed on plants exposed to *P. brassicae* oviposition (EF) compared with control plants (F) (2012: $F_{1,28} = 4.46$, $P = 0.044$; 2013: $F_{1,28} = 6.51$, $P = 0.016$; Fig. 3), but the same was not true for the pupal parasitoid *P. puparum* (2013: $F_{1,28} = 0.02$, $P = 0.883$; Fig. 3).

Table 1. Parasitoid and hyperparasitoid species and number of individual wasps emerging from *Pieris brassicae* caterpillars and pupae, and individual *Cotesia glomerata* cocoons collected from *Brassica nigra* plants during the two field seasons (2012 and 2013).

Species	**Family**	**Parasitism mode**	***n* individuals**
Cotesia glomerata	Braconidae	Primary, larval parasitoid	410
Pteromalus puparum	Pteromalidae	Primary, pupal parasitoid	137
Lysibia nana	Ichneumonidae	Secondary, hyperparasitoid	177
Baryscapus galactopus	Eulophidae	Primary, hyperparasitoid	162
Mesochorus gemellus	Ichneumonidae	Primary, hyperparasitoid	8

In 2013, hyperparasitism rates by *L. nana* were significantly higher on EF plants that had experienced oviposition ($F_{1,28} = 4.23$, $P = 0.049$; Fig. 3), whereas differences were not significant for the hyperparasitoid *B. galactopus* ($F_{1,28} = 0.023$, $P = 0.88$; Fig. 3).

The BC treatment did not significantly affect parasitism rates by either parasitoids: *C. glomerata* (2012: $F_{1,28} = 0.09$, $P = 0.768$; 2013: $F_{1,28} = 0.80$, $P = 0.371$; Fig. S2, Appendices), *P. puparum* (2013: $F_{1,28} = 0.04$, $P = 0.847$; Fig. S2, Appendices), or hyperparasitoids: *L. nana* (2013: $F_{1,28} = 0.09$, $P = 0.761$; Fig. S2, Appendices) and *B. galactopus* (2013: $F_{1,28} = 0.03$, $P = 0.863$; Fig. S2, Appendices).

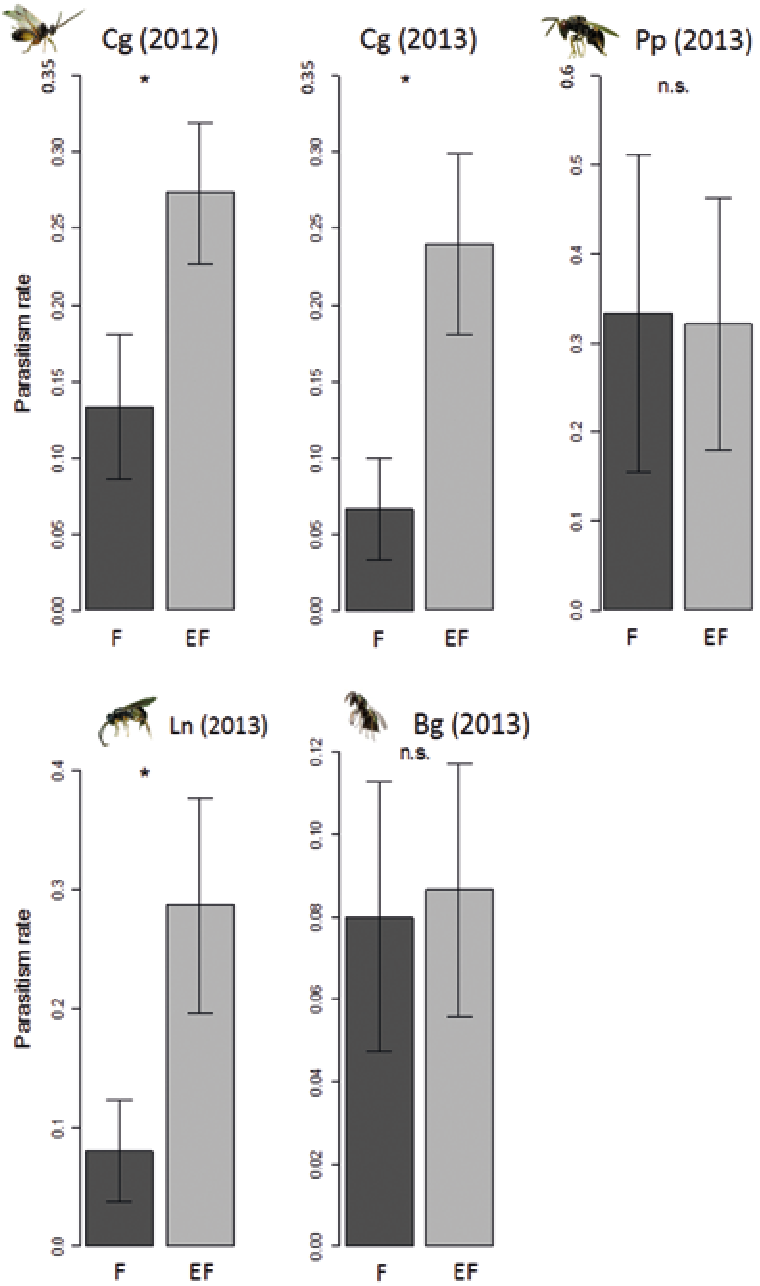

Figure 3. Plant-mediated effects of *Pieris brassicae* (Pb) oviposition on parasitism rates under field conditions, in two sequential years (2012, 2013). Bars indicate parasitism rates (mean ± SE) of *Cotesia glomerata* (Cg) larval and *Pteromalus puparum* (Pp) pupal parasitoids that developed in *P. brassicae* caterpillars or pupae respectively, *Baryscapus galactopus* (Bg) and *Lysibia nana* (Ln) hyperparasitoids that developed in *C. glomerata* larvae or cocoons respectively, on *B. nigra* previously infested with eggs (EF), or on plants infested only with caterpillars (F). Number of plants, n= 60. Asterisks indicate significant differences in mass between the different treatments ,* $P < 0.05$, ns: not significant, (ANOVA).

Effects on plant fitness

In 2012, the effect of treatments on plant fitness was assessed. Control plants (C) that had not been exposed to initial experimental herbivory by *P. brassicae* were compared with those that had been exposed to either egg deposition plus larval feeding (EF), or to larval feeding alone (F). In comparison with control plants (C), plants exposed to oviposition and caterpillar feeding (EF) produced a larger number of seeds ($\beta = 0.62$, $t_{2,48} = 2.14$, $P = 0.037$), but those that had been exposed to caterpillar feeding only (F) did not produce an increased number of seeds ($\beta = 0.27$, $t_{2,48} = 0.86$, $P = 0.396$, Fig. 4). Control plants (C) and plants exposed to oviposition plus larval feeding (EF) produced seeds of similar biomass ($\beta = -0.23$, $t_{2,48} = -0.76$, $P = 0.451$) as well as larval feeding only plants (F) ($\beta = 0.14$, $t_{2,48} = 0.44$, $P = 0.662$). Germination rates of seeds collected from clean plants, plants exposed to oviposition plus larval feeding or larval feeding only were similar ($\beta = -0.09$, $t_{2,48} = -1.059$, $P = 0.295$ and $\beta = -0.11$, $t_{2,48} = -1.12$, $P = 0.268$, respectively). Seed number and germination rate showed a significant positive correlation (Fig. S3).

None of the plant reproductive traits measured were affected by the BC treatment ($P > 0.1$). Yet, both seed weight and germination rate were significantly positively correlated with seed number (Fig. S4, S5, Appendices).

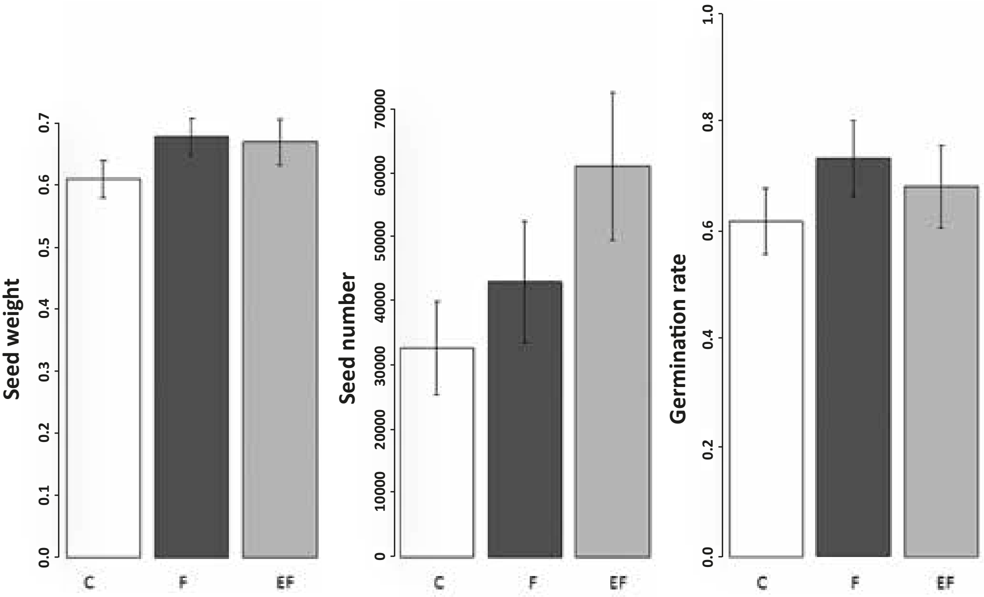

Figure 4. Plant-mediated effects of *Pieris brassicae* oviposition on *Brassica nigra* seed number, seed weight, and germination rate when plants were previously infested with eggs (EF), or only with caterpillars (F) or uninfested plants (C). Bars indicate mean ± SE of total seed weight (g), total number of seeds, germination rate, number of plants = 90 (GMM).

Discussion

Our field study showed that egg deposition might act as an "early herbivore warning" cue for the plant and that its impact cascaded up in the food chain influencing many community members from herbivores to hyperparasitoids and ultimately benefitting plant fitness. We found that changes in plant traits triggered by egg deposition of the butterfly *Pieris brassicae* reduced the biomass of the herbivore and carnivores at higher trophic levels (i.e. parasitoids and hyperparasitoids). Induction by egg deposition also affected parasitism rates of the herbivore for both the larval parasitoids and for one of their associated hyperparasitoids. It is remarkable that egg deposition at the beginning of the season, in itself not causing plant tissue damage, altered plant quality and thereby influenced performance and parasitisation rate of parasitoids and hyperparasitoids much later in the season.

Such cascading effects at different trophic levels of the food chain have previously been shown for when direct damage was inflicted by herbivore feeding (Rasmann & Turlings 2007, Soler *et al.* 2007, Harvey *et al.* 2003, Bukovinszky *et al.* 2008, Poelman *et al.* 2012). We demonstrated that plant-mediated responses to oviposition could have strong season-long effects on the focal herbivore and its associated parasitoid community. These effects ultimately affected plant fitness: in comparison to control plants, those that had experienced oviposition produced a larger number of seeds. This is unexpected because herbivores and plants have an antagonistic interaction so that the former could negatively influence the fitness of the latter (Karban & Baldwin 1997). All plants were exposed to the naturally occurring insect community; control plants received natural herbivore infestation and test plants had received an additional herbivory or chemical elicitor treatment at the start of the experiment. We expected that plants, which had not been experimentally exposed to *P. brassicae* oviposition, but were exposed to all other community members, would yield larger numbers of seeds than the egg-infested plants.

This expectation was based on the fact that *P. brassicae* caterpillars are voracious florivores that can reduce plant biomass substantially (Smallegange *et al.* 2007). Here, we first discuss (a) how plant-mediated effects of egg deposition cascade through the food chain, then (b) how they contribute to direct and indirect defenses and finally (c) provide a hypothesis on why plants previously exposed to egg deposition had a larger seed production than untreated plants.

Plant-mediated effects of egg deposition in a multitrophic context

We provide evidence that different developmental stages of the same herbivore species can elicit differential effects on higher trophic levels of the same food chain. As for the plant-herbivore interaction, egg deposition by *P. brassicae* butterflies negatively influenced the biomass of *P. brassicae* larvae as found in other insect-plant systems (Hilker & Fatouros 2015). In interactions between Scots pine (*Pinus sylvestris*) and pine sawfly (*Diprion pini*), plant-mediated effects of egg deposition were even shown to reduce fecundity of the developing females. Thus, these effects negatively influence the performance of the next herbivore generation (Beyaert *et al.* 2011). Furthermore, evidence from the family of Brassicaceae indicate that the “early herbivore warning” strategy might be more common in plants than was previously thought (Pashalidou *et al.* 2015b). To date the consequences of these plant-quality changes on the performance of organisms at higher trophic levels have received little attention. We found that, under natural conditions, the plant-mediated effects of egg deposition cascaded from the herbivore to its associated larval parasitoid *Cotesia glomerata* at the third trophic level and the hyperparasitoids *Lysibia nana* and *Baryscapus galactopus* at the fourth trophic level*.* Although not related to egg deposition such cascading effect reaching up to the fourth trophic level has previously been found for effects of belowground herbivory (Soler *et al.* 2005). Interestingly this study showed that parasitism rates of *C. glomerata* and *L. nana* were also increased on plants that had experienced the oviposition event. Potential explanations for such a pattern may involve the parasitoids using information from the plant after egg deposition. Egg deposition is known to induce plant volatiles that lure not only egg parasitoids, but also larval parasitoids towards the plant (Tamiru *et al.* 2011, Fatouros *et al.* 2012, Pashalidou *et al.* 2015a).

The increased parasitism rates of parasitoids in hosts fed on plants that were previously exposed to eggs, which is associated with a reduced offspring weight, is indicative of a trade-off affecting parasitoid fitness. In a previous study, though, we found that egg deposition might alter plant volatile blends upon caterpillar feeding within the hours immediately after caterpillars hatch from eggs. The parasitoid *C. glomerata* exploited the volatile changes that were induced by egg deposition to locate the early stages of its host. This host-searching efficiency of the wasps was positively correlated with host quality in terms of parasitism success and parasitoid performance (Pashalidou *et al.* 2015a). Hyperparasitoids also use chemical information from the plant to locate their hosts, and show odour-based preferences similar to those of their host *C. glomerata* (Poelman *et al.* 2012). In future field studies it would be interesting to test whether specialist hyperparasitoids (e.g. *L. nana*) might mark their hosts and return when the larvae of *C. glomerata* are about to egress from *P. brassicae* caterpillars. This fascinating behaviour has also been observed in the egg-larval parasitoid *Hyposoter horticola,* which monitors and marks the location of multiple possible hosts (e.g. eggs of the butterfly *Melitaea cinxia*) until the hosts become suitable for parasitism (van Nouhuys & Kaartinen 2008).

Plant-mediated effects of egg deposition on direct and indirect defences

The present study allowed the teasing apart of caterpillar-mediated effects from egg-mediated plant effects under natural conditions. In terms of their effects on the plant and its associated insect community, it is revealed that both stages need to be considered. Previous studies have shown that egg deposition can provide information on upcoming herbivory but only in the laboratory (Beyaert *et al.* 2011, Pashalidou *et al.* 2013, Geiselhardt *et al.* 2013, Pashalidou *et al.* 2015b). Besides direct defences, egg-associated elicitors might also trigger indirect defences (Pashalidou *et al.* 2015a), and here we provide evidence for both routes in a community-wide context. Direct and indirect defences induced by egg deposition have been shown to act synergistically in reducing herbivore pressure in plants (Fatouros *et al.* 2014).

The mechanistic basis of plant-mediated interactions between insects has been extensively studied (Stam *et al.* 2014), but relatively little is known about the mechanisms governing oviposition-mediated changes in plant defences. Up to now, it is known that plants activate a response directed against the eggs that is similar to recognition of pathogens (pathogen-associated molecular patterns) and involves the activation of systemic acquired resistance (Reymond 2013, Hilfiker *et al.* 2014). Here, we aimed to gain a better understanding of the effects of egg-associated elicitors by exploring the effect of the male butterfly-derived anti-aphrodisiac benzyl cyanide (BC). Benzyl cyanide was found to be present in minute amounts in the egg-associated secretion used to attach the eggs of *P. brassicae* to the leaf surfaces. Benzyl cyanide has been shown to act as a chemical elicitor of plant responses that induces phenotypic and transcriptomic changes in *Brassica oleracea* plants (Fatouros *et al.* 2008). In our study, BC affected caterpillar biomass so it may play a role on direct defences. However, its role on the production of plant cues that cause increased parasitism rates remains to be elucidated. Since the direct defence effect did not cascade to higher trophic levels, it is likely that BC triggered a weaker response than egg deposition, or maybe other yet unknown compounds are playing a role.

Plant-mediated effects of egg deposition on plant fitness

Our data showed that plants exposed to egg deposition as well as feeding caterpillars produce a higher number of seeds than plants that were not exposed to herbivory. Here, we propose possible scenarios regarding plant compensation of fitness loss due to herbivory based on the predictions from the current literature. Firstly, reproductive escape of *B. nigra* has been suggested as a strategy to prevent fitness loss resulting from herbivore damage (Lucas-Barbosa *et al.* 2013). Upon egg deposition by *P. brassicae*, plants reallocate their resources to accelerate seed production while maintaining interactions with carnivores and pollinators to the benefit of plant fitness (Bruinsma *et al.* 2014, Lucas-Barbosa *et al.* 2013, Lucas-Barbosa, unpublished data).

A second possible explanation for our results comes from herbivore-induced plant defences that may result in resistance to a subsequently arriving herbivore (Kessler & Baldwin 2004: the authors termed this phenomenon as vaccination). Tobacco plants (*Nicotiana attenuata*) infested with the myrid bug *Tupiocoris notatus*, increase their direct and indirect plant defences against subsequent attack by *Manduca sexta* hornworms, and hornworms avoid plants infested with myrids (Kessler & Baldwin 2004). The combination of direct and indirect defences on myrid-attacked plants resulted in a significant plant fitness advantage in environments where both herbivores were present. This may suggest why egg-infested plants in our study had increased seed numbers compared to control plants. The increased reproductive output of plants experiencing oviposition might reveal that plant responses against *P. brassicae* reduced the likelihood of colonisation by other herbivores such as *Plutella xylostella.*

Finally, Agrawal (2000) proposes (over-)compensation phenomena by which damaged plants might increase their fitness compared to undamaged ones. Plants might phenologically separate their reproductive resources to pre-herbivory and post-herbivory based on a cue that can be used to predict high risk of herbivory. If plants perceive this herbivory cue then they increase their reproduction rate (Agrawal 2000). Field experiments showed that natural herbivory by large grazers (i.e. mule deer, *Odocoileus hemionus*, and elk, *Cervus elaphus*) on scarlet Gilia (*Ipomopsis aggregata*) caused increased seed production compared to undamaged plants (Paige *et al.* 1999). Gentian plants (*Gentianella campestris*) increased fruit production after clipping (i.e. removing half of the biomass), compared to unclipped plants (Lennartson *et al.* 1998). These phenomena might be valid under a set of resource- and environmental conditions (Trumble *et al.* 1993, Strauss & Agrawal 1999, Agrawal 2000). Among these conditions were proposed high predictability of herbivory (e.g. predictable herbivores upon deposition of eggs in our case), and optimal environmental conditions (e.g. high light and water levels & high soil fertility) that are likely to apply to our model system.

Conclusions

Overall, our study highlights that plants can use egg deposition as an "early herbivore warning" cue under natural conditions. Egg deposition induces changes in plant quality that affect insects at higher trophic levels in the food chain. Previous research has demonstrated that egg deposition may induce direct plant resistance against subsequently feeding larvae in the laboratory. Our study extends this framework to an ecologically relevant context of a diverse parasitoid and hyperparasitoid community associated with a focal herbivore in the field. We found that direct and indirect defences act in concert, triggered by egg deposition by a voracious herbivore. In natural communities, individual insect species may respond differentially to oviposition-induced plant responses, and likely, the ecological outcome can vary depending on specific insect species and traits. These results emphasise the importance of egg deposition as warning cue for the plant to anticipate future herbivory under natural conditions and add an important dimension to our understanding of phenotypic plasticity of induced plant defences. Strikingly, egg deposition mediates these defences against subsequently feeding caterpillars, and increases plant fitness and this may impact the structure and ecology of the native insect community up to the fourth trophic level.

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Chapter 6


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General discussion

Foteini G. Pashalidou

Plants live in complex communities, which comprise pathogenic and symbiotic microorganisms such as fungi and bacteria, herbivores and their natural enemies such as predators and parasitoids, and pollinators. Every plant is a member of a speciose insect community that involves tens to hundreds of species (Stam *et al.* 2014). About half of the known insect species have evolved to feed on plants. Some of these herbivorous species are severe pests that destroy annually one fifth of the total world crop production (Sallam 2012). Plants have defences to cope with a diverse community of herbivorous insects. A plants' chemical, physiological, or morphological phenotype is plastic and can change in response to herbivory. These changes can affect subsequent interactions with herbivores and other plant-associated insects. Plant phenotypic changes may vary with the type of attackers. Different types of herbivorous insects such as chewing or sucking insects may induce different plant responses because of differences, for example, in wounding or specific elicitors. Plenty of literature describes how plants defend themselves against their insect herbivore attackers (see reviews by Karban & Baldwin 1997, Dicke & Hilker 2003, Agrawal 2007, Kessler & Halitschke 2007, Poelman & Dicke 2014).

Plants can perceive insect attack during feeding as well as when eggs are deposited. The *aim* and *the originality* of this thesis was to unravel whether plant responses mediated by egg deposition affect subsequent herbivore stages and extend the interaction to other plant-associated insect community members of the food chain.

There is ample knowledge on egg-induced plant defences that harm the eggs directly and/ or indirectly (Hilker & Meiners 2011, Hilker & Fatouros 2015). There is, however, limited information on a plant's ability to perceive insect eggs as a cue for upcoming herbivory. In this thesis, I have evaluated the ecological effects of plant responses to insect eggs as a cue that indicates future feeding damage and the role of these responses on induced plant defences against herbivory. Experiments were conducted both in the laboratory and in a (semi-) field set-up, from the herbivores' and plants' perspective.

The main questions that I addressed were: (a) how specific were plant responses against egg deposition of different lepidopteran species (specificity of induction), and (b) what were the effects on the performance of subsequently feeding larvae (chapter 2). I extended the number of plant species to test (c) how common and persistent the plant-mediated effects of egg deposition were in the Brassicaceae family (chapter 3). Plant-insect egg interactions were also evaluated to test (d) how they affect the preference and performance of parasitoids of the third trophic level in the laboratory and parasitoids and hyperparasitoids up to the fourth trophic level in a natural community context (chapters 4 and 5) (Fig. 1). Consequently, these results may improve our understanding of early herbivore alert responses and their role in the evolution of induced plant defences.

In this chapter, I discussed the main findings of this thesis and linked them with the current literature. This thesis project took a multidisciplinary approach bringing together ecological, physiological, and chemical aspects of plant - insect egg interactions mainly between the annual weed *Brassica nigra*, the Large Cabbage White butterfly *Pieris brassicae*, and its associated parasitoids and hyperparasitoids. My aim was to place the results of this thesis into a wider framework on the ecology and evolution of herbivore–induced plant responses.

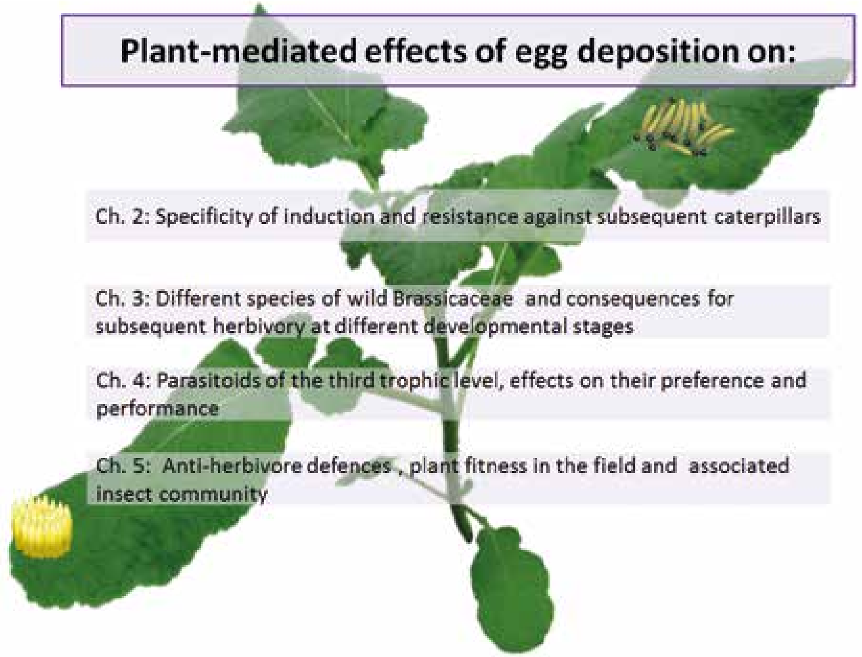

Figure 1. Overview of the experimental chapters of this thesis and their biological links regarding plant-mediated effects of egg deposition.

Specificity of anti-herbivore responses induced by egg deposition

Plants can respond to insects egg deposition by directly killing their eggs, as has been shown for more than 20 plant species (Hilker & Fatouros 2015). Thus, plants can minimize the effect of feeding damage by reducing the survival of an early non-feeding stage of herbivores (Hilker & Fatouros 2015). We can differentiate between plant responses to eggs that target the egg stage itself directly, such as the formation of necrotic tissue below the eggs that leads to eggs desiccating or dropping off the plants (hypersensitive response (HR)-like necrosis) or indirectly, such as by the attraction of egg parasitoids. Moreover, plants can exploit information associated with egg deposition as a cue and respond with phenotypic changes that affect subsequently feeding insects either directly by reduction on herbivore performances and fecundity or indirectly by early attraction of larval parasitoids.

Here, I focused on addressing the question whether plants can use insect eggs as warning cues to mount defences against subsequently feeding herbivores and how specific these responses can be. These egg-induced responses can result in changes in plant quality and cause reduced biomass of developing larvae and pupae (Pashalidou *et al.* 2013). Until now, such effects have been shown in four different plant-herbivore systems, i.e. (a) pine (*Pinus sylvestris*)-pine sawfly (*Diprion pini*) (Beyaert *et al.* 2011), (b) Brassicaceae- *P. brassicae* (Beyaert *et al.* 2011, Geiselhardt *et al.* 2013, Pashalidou *et al.* 2013, Pashalidou *et al.* 2015), (c) tobacco plant (*Nicotiana attenuata*)- beet armyworm (*Spodoptera exigua*) (Bandoly *et al. submitted*) and (d) the field elm *(Ulmus minor)*- elm leaf beetle (*Xanthogaleruca luteola*) (Austel *et al*. *unpublished data*). Interestingly, plants can recognise and respond to egg deposition by some herbivores but not others (Table 1, Fig. 2).

For example, *B. nigra* plants exposed to *P. brassicae* eggs mount anti-herbivore defences against subsequent attackers. However, when plants were exposed to *M. brassicae* eggs, they did not show enhanced defences against subsequently feeding larvae (Pashalidou *et al.* 2013). In *Arabidopsis thaliana* it was shown that, when leaves were treated with egg extract of *P. brassicae,* the biomass of *P. brassicae* larvae was unaffected upon feeding on the same plant (Bruessow *et al.* 2010).

On the other hand, when *A. thaliana* plants were treated with extracts of eggs of *P. brassicae* or *Spodoptera littoralis*, the subsequently feeding larvae increased their biomass (Bruessow *et al.* 2010). In tobacco, *N. attenuata*, plants respond to the eggs of the abundant generalist *S. exigua* with changes that negatively affect subsequently feeding larvae. However, egg deposition of tobacco hornworm *Manduca sexta* seems not to affect the subsequent feeding larvae (Bandoly *et al. unpublished data*). The specificity of induction may be due to species-specific egg-related elicitors that bind with plant receptors and trigger responses (Reymond 2013). It is elusive which kind of mechanism underlies these differences in plant responses and why some eggs are recognised by the plants and others not. Here, I present three possible strategies regarding plant recognition of egg deposition and elicitation of plant defences (Fig. 2):

(1) Plants recognise egg depositions by specific herbivores and exploit these as warning cues for high risk of herbivory and mount defences against subsequent developmental stages of these herbivores. In a recent review it was proposed that plants could particularly recognize and respond to specific herbivores that

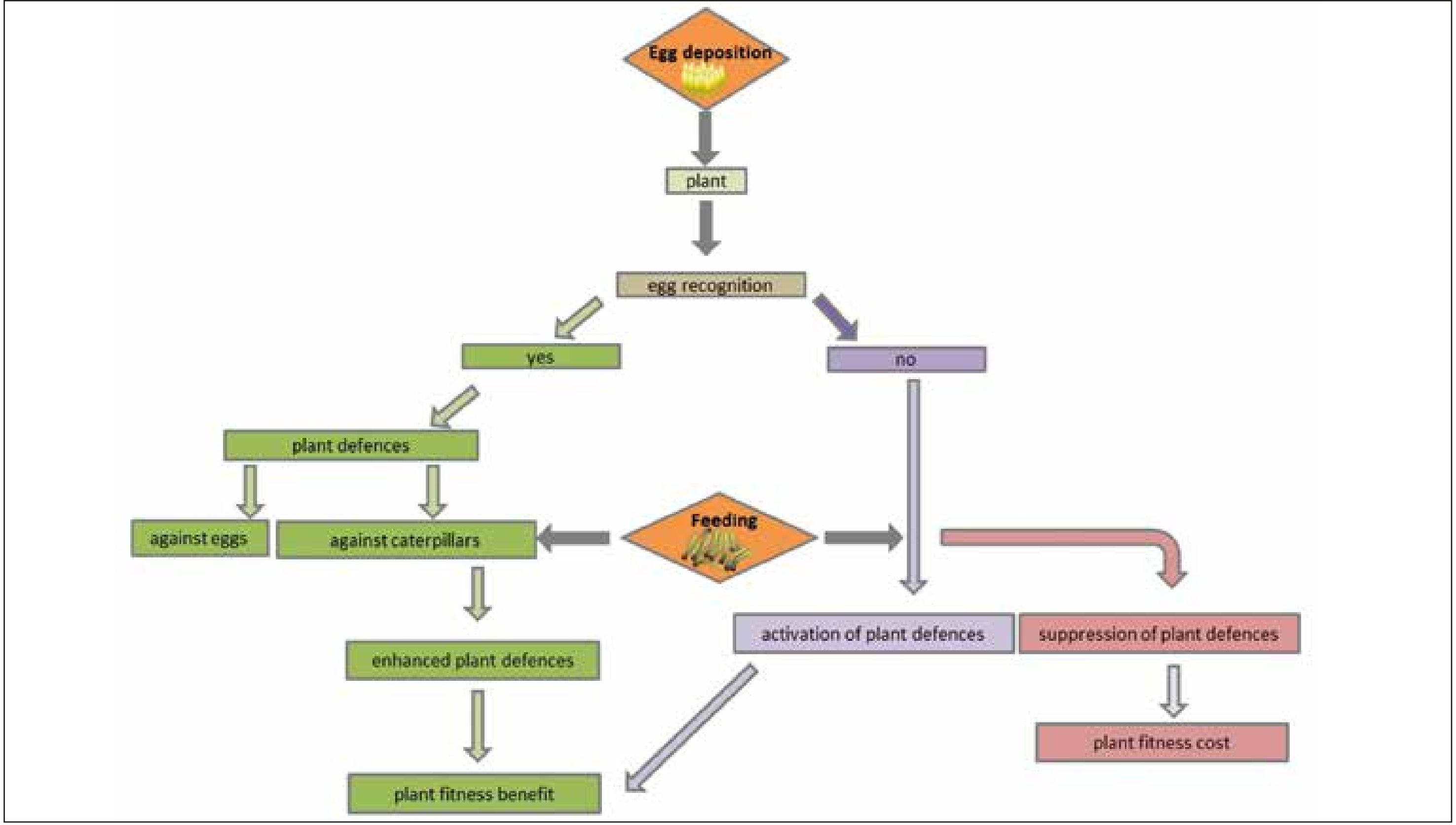

Figure 2. Simplified conceptual model of plant responses to egg deposition in plant-herbivore interactions. Plants can recognise eggs of certain insect species and respond by either mounting egg-killing defences (e.g. hypersensitive response-like necrosis, attraction of egg parasitoids) or defences against subsequently feeding larvae. Plant-mediated effects due to oviposition affect subsequent herbivores either directly (by decreased performance and fertility) or indirectly (by early attraction of larval parasitoids) and eventually decrease the negative impact of herbivory on plant fitness. Alternatively, plants do not recognise the eggs or specific herbivores may suppress plant responses against oviposition as a counter-adaptation strategy.

Table 1: Overview of studies testing plant-mediated effects of egg deposition on subsequent herbivores.

Plants	Life history	Herbivore Eggs	Herbivore larvae	Larval biomass	Reference
Brassicaceae					
Arabidopsis thaliana	annual	*P. brassicae*	*P brassicae*	negative	Geiselhardt et al. 2013
Arabidopsis thaliana	annual	*P. brassicae**	*P brassicae*	no effect	Bruessow et al. 2010
Arabidopsis thaliana	annual	*P. brassicae**	*S. littoralis*	positive	Bruessow et al. 2011
Brassica nigra	annual	*P. brassicae*	*P. brassicae*	negative	Pashalidou et al 2013
Brassica nigra	annual	*P. brassicae*	*M. brassicae*	negative	Pashalidou et al 2013
Brassica nigra	annual	*M. brassicae*	*M. brassicae*	no effect	Pashalidou et al 2013
Brassica nigra	annual	*M. brassicae*	*P. brassicae*	no effect	Pashalidou et al 2013
Brassica oleracea	biennial	*P. brassicae*	*P. brassicae*	negative	Pashalidou et al 2015
Moricandia arvensis	annual	*P. brassicae*	*P. brassicae*	no effect	Pashalidou et al 2015
Moricandia moricandioides	annual	*P. brassicae*	*P. brassicae*	negative	Pashalidou et al 2015
Sinapis arvensis	annual	*P. brassicae*	*P. brassicae*	negative	Pashalidou et al 2015
Solanaceae					
Nicotiana attenuata	annual	*S. exigua*	*S. exigua*	negative	Bandoly et al (*submitted*)
Pinaceae					
Pinus sylvestris	perennial	*D. pini*	*D. pini*	negative	Beyaert et al 2011

*supernatant of aqueous extract of *P. brassicae* eggs have strong impact on plant fitness (Ali & Agrawal 2012). Plants need to find a balance between defence and reproduction (Schoonhoven *et al.* 2005). Selection pressure is high, because plants require a maximal reproductive success and at the same time must defend themselves against herbivores. Therefore, plants may have evolved to recognise reliable cues, such as associated with the deposition of herbivore eggs that predict future herbivory and respond with the induction of defences before and upon feeding. The different studies presented in Table 1 actually fit in this concept of plant-mediated responses to oviposition showing that, upon egg recognition, plants defend themselves more effectively against larvae with negative consequences for the development of the herbivore. Interestingly, for some herbivores negative effects were found even in the next generation (Beyaert *et al.* 2011). In addition, these enhanced plant defences induced by oviposition might indicate priming (Bandoly *et al. submitted*). Priming of defences in plants by a first stress enables them to respond stronger and faster against a second stress. *Nicotiana attenuata* plants are primed by egg deposition of *S. exigua* and respond stronger upon feeding activities with increased production of defensive traits such as trypsin protease inhibitors (TPIs) (Bandoly *et al. submitted*, Table 1).

From the studies presented in Table 1, specificity of induction by egg deposition against herbivores seems not to be related with herbivore specialisation and plant life-history strategies. *Brassica nigra* and *Nicotiana attenuata* plants recognised egg deposition by specialist and generalist herbivores such as *P. brassicae* and *S. exigua* respectively. Both herbivores are very abundant on their host plants, and likely to exert strong selection: *P. brassicae* is one of the most important lepidopteran herbivores on Brassicaceae, including *B. nigra* (Smallegange *et al.* 2007), and *S. exigua* is the second most important lepidopteran herbivore on tobacco plants (Rayapuram & Baldwin 2007). Therefore, their eggs may represent a reliable cue for *B. nigra* and *N. attenuata* plants, respectively, to respond against the feeding caterpillars and reduce the negative impact of herbivory on the plant. Interestingly, annual, biennial, and perennial plants (e.g. trees) are able to respond to egg deposition and to feeding herbivores. In the case of the tested wild Brassicaceae plants, egg deposition induced plant defences that affect different developmental stages of the herbivores such as larvae and pupae of the same generation (Pashalidou *et al.* 2015). For the tree species tested (pines), the plant-mediated effects of egg deposition were also affective against the next generation of insects (Beyaert *et al.* 2011, Table 1). This might be correlated with the reproductive strategy of perennial plants. Perennial plants are those with long life expectancy and low energy invested in reproduction, whereas annual plants have a short life expectancy and high reproductive effort (Pianka 1970, Grime 1977). It might be important for plants like trees to deal with herbivory in the long run, especially when the herbivores have many generations. For annual plants, it might be important to deal with herbivory on a short term since their reproduction is restricted to one generation.

(2) Plants may not need to evolve defences against all herbivore eggs because their defences induced by feeding caterpillars, e.g. toxins, are strong enough to combat the herbivores in this phase. This may apply to herbivores like the cabbage moth *M. brassicae*. *Mamestra brassicae* caterpillars suffer from high levels of toxins, i.e. glucosinolates, in wild brassicaceous plants such as *B. nigra*. As soon as the larvae feed on the plants, the plant produces mainly the glucosinolate sinigrin, which results in the caterpillars leaving the plants quickly (Hopkins *et al.* 2009). Therefore, the wild crucifer *B. nigra* does not suffer considerable damage due to *M. brassicae*. For *M. sexta,* in contrast, indirect plant defences may be more effective against the caterpillars in *N. attenuata* than direct plant defences (Halitschke *et al.* 2000, Kessler & Baldwin 2001) and this may explain why no effect on subsequent caterpillars was found when plants were previously exposed to oviposition by *M. sexta* (Bandoly *et al. unpublished data*). In the same system it has been already shown that *M. sexta* can circumvent plant responses in *N. attenuata* by suppressing the wound-inducible nicotine production (Voelckel & Baldwin 2004). In addition, plants may not need to recognise insect herbivore eggs that are not the main herbivores imposing selection on plant defences. For example, *P. brassicae* is not very abundant on *Moricandia arvensis* plants but instead larger grazers such as sheep and goats consume large amounts of plant biomass (Gómez 1996).

(3) Herbivores may develop counter-adaptations to egg-induced plant defences and suppress them. As shown in *A. thaliana*, plants treated with egg extract of *P. brassicae* showed increased accumulation of salicylic acid (SA) at the oviposition site, which negatively interacted with jasmonic acid (JA)-mediated defences (Bruessow *et al.* 2010). The phytohormones SA and JA are two major players that regulate the main signal transduction pathways that control induced plant defences (Pieterse & Dicke 2007). Application of *P. brassicae* egg extract suppressed the induction of insect-responsive genes after caterpillar feeding. This study suggests that insects may evolve ways to circumvent induced plant defences by releasing specific egg-derived elicitors during egg deposition that suppress them (Bruessow *et al.* 2010).

Plant - insect egg interactions in a multi-trophic context

The novelty of this project lies in investigating whether plants use eggs as an early warning cue to predict high risk of herbivory. Interestingly, it was found that plants indeed use eggs from specific species as a warning cue and that plant-mediated responses against oviposition affected herbivores (chapters 2, 3, 5) and carnivores up to higher trophic levels (chapters 4, 5) in a single food chain and ultimately increased plant fitness (chapter 5).

These results indicate the need to re-evaluate models regarding plant-mediated species interactions, since egg deposition may influence the outcome of these interactions.

In a broader context of plant-mediated interactions there are still many open questions regarding how these interactions affect the structure of insect communities and to what extent plant phenotypic plasticity and trophic cascades contribute to community dynamics (Poelman & Dicke 2014). Field studies on early-arriving herbivores and subsequent colonizers indicate that indirect plant-mediated interactions might be as important as trophic interactions to structure insect communities (van Zandt & Agrawal 2004, Viswanathan *et al.* 2005, Bukovinszky *et al.* 2008). It would be interesting to test how plants alter their phenotype upon egg deposition and subsequent herbivory, and how this affects other herbivore species. Such indirect interactions among herbivore species are quite common in a natural community context (Fig. 3) (Utsumi *et al.* 2011).

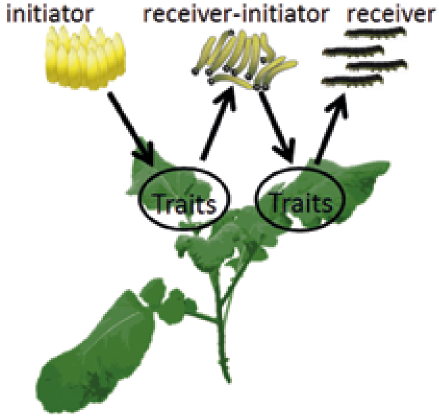

Figure 3. Plants may alter their phenotype in response to egg deposition, affecting interactions with subsequent herbivores. Egg deposition (initiator) may induce a plant response that affects the hatching herbivore species (receiver) and in its turn may differentially affect similar or different plant traits that are received by a second herbivore species etc. The figure is an extended version of a model regarding indirect interactions among herbivores (Utsumi *et al.* 2011).

Up to now, no studies on plant-insect egg interactions have taken into account indirect plant mediated interactions in a community context with more than one herbivore. An important question related to these indirect plant-mediated interactions is whether specific herbivores that elicit induced plant responses could strongly affect other herbivores or whether particular herbivores are more resistant to induced plant defence (Denno *et al.* 1995, Denno *et al.* 2000, Poelman & Dicke 2014).

While herbivores feed, a plant's phenotype is changing and these changes may influence plant quality (Agrawal 2000, Poelman *et al.* 2008). However, a plant's phenotype that changes because of egg induction should be added to these models since plant - insect egg interactions can play a major role in the specificity of induction and consequently in the resistance or susceptibility of the induced plant phenotypes to subsequent multiple herbivory (Fig. 3). This model can be even further extended, involving multiple herbivores and their parasitoids that form a complex network. Plant-mediated species interactions have important effects on insect community organisation within natural ecosystems. In a recent study, it was shown that flowering *B. nigra* plants are able to compensate for herbivory by *P. brassicae* only when the interactions with carnivores are maintained (Lucas-Barbosa 2015). Egg deposition represents the beginning of a new herbivore generation, and since any induced response may potentially affect all other members of the community and may cascade to higher trophic levels over a season, it is an essential part of plant-mediated species interactions.

Conclusions and future directions

Egg deposition is a stage of herbivore-plant interactions that has been ignored in research until recently. However, an increasing number of studies show that plants recognise specific herbivore eggs and respond accordingly. The research presented in this thesis demonstrates that plants such as *B. nigra* develop anti-herbivore resistance when exposed to the eggs of *P. brassicae* and not by another lepidopteran species (*Mamestra brassicae*).

These results suggest specificity of plant responses to particular herbivore species (chapter 2). In addition, when extending the number of brassicaceous species, I found that apart from *B. nigra,* other wild Brassicaceae species also respond to egg deposition by mounting defences against subsequently feeding caterpillars (e.g. *Sinapis arvensis, Brassica oleracea*) (chapter 3). This indicates that such a plant-mediated effect of egg deposition might be a common trait across wild brassicaceous plants.

To further evaluate the effects of these responses at higher trophic levels, it was tested whether egg deposition could actually change the emission of herbivore-induced plant volatiles over time and whether these changes were correlated with the behavioural responses of larval parasitoids (chapter 4). Indeed, the wasps used these changes to locate just-hatched caterpillars that represent a better quality host. Parasitism success and wasp performance confirmed that there is a fitness benefit for wasps to use oviposition-induced plant volatiles to locate their hosts. The last experimental chapter of the thesis (chapter 5) highlights those plant-mediated effects of egg deposition and their consequences for plant defences, on higher trophic levels and ultimately on plant fitness.

Interestingly, egg deposition mediates plant defences directed against subsequent caterpillars and indirectly by enhancing the attraction of parasitoids. Ultimately, egg deposition also influenced plant reproduction and resulted in increased seed numbers. The results from this thesis emphasise that plant-insect egg interactions should be taken into account in future studies. Importantly, such responses add another level to adaptive plasticity of plant responses against herbivores starting at the initial phase of colonization, even before the actual feeding damage. Plants use a wide range of responses against herbivory and against insect egg deposition. These responses are often species-specific with regard to the herbivores and the plants. In future, more field studies are needed to elucidate the ecological effects of oviposition with respect to plant damage, and egg-laying behaviour (eggs laid singly or in clusters) and ultimately plant fitness. Changes in plant phenotype induced by oviposition might be common in the Brassicaceae family and more plant species should be tested to identify how strong these interactions are under natural conditions and whether they benefit plant fitness.

At the mechanistic level, it would be interesting to identify egg-associated elicitors and corresponding plant receptors, since plant responses to particular herbivores are species specific. Furthermore, it has been shown that egg deposition may enhance direct or indirect plant defences against subsequent herbivory. However, the mechanisms that underlie such responses in the Brassicaceae remain poorly understood. For the future, in the system of *Brassica nigra –P. brassicae* we can speculate that we might expect priming of anti-herbivore defences with higher expression of defence-related genes, upon feeding compared to gene expression in plants that are exposed only to feeding and not receive oviposition.

This hypothesis could be addressed because oviposition negatively affected caterpillar performance compared to caterpillars that developed on egg-free plants. To date, there are only two molecular studies showing actual priming of plant responses to oviposition in the context of resistance against subsequent herbivory. A first study was performed on tomato plants (*Solanum lycopersicum*) showing that plants previously exposed to egg deposition by *Helicoverpa zea* increased JA (jasmonic acid) biosynthesis and displayed a stronger expression of defence-related protease inhibitor (*PIN2*) upon subsequent herbivory, compared to plants that had not received eggs previously (Kim *et al.* 2012). However, Kim *et al.* (2012) did not measure caterpillar performance. A second very recent study was performed in *N.*

attenuata (Table 1). Insect egg deposition by *S. exigua* enhanced concentrations of secondary plant metabolites and resulted in increased larval mortality on plants exposed to oviposition compared to plants exposed to larval feeding only (Bandoly *et al. submitted*).

Chemical elicitors and plant strengtheners or mixtures of compounds that enhance plant resistance against herbivores gain attention in the last years as a part of integrated pest management in agriculture (Tamiru *et al.* 2012, Sobhy *et al.* 2014, Tamiru *et al.* 2015). For maize (*Zea mays*) it has been shown that treating the plants with a chemical mimic of SA (BTH), results in induced resistance to a wide range of diseases and the BTH-treated maize after caterpillar infestation were highly attracted to natural enemies compared to untreated plants (Friedrich *et al.* 1996, Tally *et al.* 1999, Rostás & Turlings 2008). A few studies including chapter 5 of this thesis, have tested the effects of chemical elicitors or plant strenghteners in the field in relation to attraction of parasitoids and predators.

In general, it remains unclear which compounds should be enhanced to improve the attraction of natural enemies and to increase the level of direct resistance against specific pests (Sobhy *et al.* 2014). Egg deposition can provide a warning signal for the plants that indicates high risk of herbivory, and it has been shown in the field that this could result in increased plant resistance against feeding herbivores and in increased parasitisation rates. Future research on possible elicitors that induced these plant responses under field conditions is highly recommended since their use may enhance the presence and efficiency of biological control agents and improve plant resistance.

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Summary

Plants are members of species-rich insect communities and the majority of these species are herbivores. The fact that ca. 50% of the known insect species feed on plants suggests that plants are under strong selection pressure to respond against insect herbivores. Plants have evolved fascinating defences against herbivory. These defences are broadly separated into constitutive when they are constantly expressed in plants and induced when they are activated upon herbivory. In this thesis, I focused on induced plant defences. Induced plant defences can be further divided in direct and indirect defences. Direct defences negatively affect herbivore behaviour or performance, while indirect defences promote the effectiveness of the natural enemies of the herbivores such as predators and parasitoids. Since more than twenty years it has been shown for many plant-herbivore systems that different herbivores can induce different phenotypic changes in plants. These changes can differentially affect subsequent attackers and their associated insect community members. Many studies of induced plant defences consider feeding damage as the first interaction between plants and insects. However, egg deposition precedes feeding in many herbivores and to date there are several studies that describe how some plants kill the eggs directly or how plants respond to eggs e.g. by attracting predators or parasitoids that cause egg mortality. However, only very few studies have shown that eggs could act as a “warning cue” for the plant, which could indicate upcoming herbivory.

The *aim* of this study was to investigate whether *egg deposition can act as warning cue* for the plant by modifying its responses against subsequently feeding caterpillars. Furthermore, it was investigated whether plant-mediated responses to oviposition affect later developmental stages of the herbivore such as larvae and pupae, and their associated parasitoids. The investigations were extended to four more plant species to test how common plant - mediated effects of oviposition on subsequent developmental stages of the herbivore are. Furthermore, the effects of egg deposition on plant responses to subsequent herbivory were tested in a laboratory set-up, a semi-field set-up and in a field experiment. The main plant species that was used for most of the experiments was the black mustard *Brassica nigra. Brassica nigra* is an annual weed belonging to the Brassicaceae family and is native in The Netherlands. It grows in riverine areas and it is known for high amounts of secondary metabolites, i.e. glucosinolates, which play an important role in defences against insect herbivores.

To better understand induced plant defences it is important to use wild plant species since their defence traits are not modified by artificial selection, as is the case for many crops. Insect herbivores associated with brassicaceous plants, such as the Large Cabbage White *Pieris brassicae*, are well studied. Therefore, Brassicaceae-Pieridae interactions represent a suitable system to address fundamental questions

regarding induced plant defences. *Pieris brassicae* was the main herbivore used during the present research project; it mainly feeds on plants in the Brassicaceae family. As a specialist, *P. brassicae* has evolved adaptations to detoxify glucosinolates and besides being folivorous, it can feed on the flowers. I extended the study on the effects of plant-mediated responses to oviposition from herbivores to parasitoids, by focussing on the gregarious larval parasitoid *Cotesia glomerata*. *Cotesia glomerata* was previously shown to use herbivore-induced plant volatiles to locate its hosts, *Pieris* caterpillars. Here, I also tested whether *C. glomerata* wasps use plant volatiles induced by oviposition to locate just-hatched caterpillars. Finally, interactions between plants and *P. brassicae* eggs were tested in a field experiment. During the field experiment, plants were infested with eggs and I tested whether oviposition affects the performance of herbivores and carnivores at higher trophic levels, i.e. parasitoids and hyperparasitoids.

My research showed that *B. nigra* plants recognise the eggs of *P. brassicae* butterflies and respond with phenotypic changes that affect subsequent developmental stages of the herbivore such as larvae and pupae. Results found in the laboratory and under field conditions were similar. Plant responses to oviposition were species-specific: plants responded only to the eggs of *P. brassicae* and not to eggs of the generalist moth *Mamestra brassicae*. Furthermore, also other Brassicaceae tested (*Brassica oleracea, Sinapis arvensis, Moricandia moricandioides*) were found to respond to *P. brassicae* eggs and exhibited defences against subsequent caterpillar feeding. These results indicated that plant responses against egg deposition might be more common in nature than was thought before. Furthermore, carnivorous insects such as *C. glomerata* parasitoids were able to use herbivore-induced plant volatiles altered by previous oviposition. This behaviour of the wasps was associated with higher fitness: parasitism success is higher when the wasps parasitised very young caterpillars compared with older ones.

Similarly, in the field plants infested with eggs attracted more larval parasitoids compared with plants infested with caterpillars only.

Apart from parasitoids, hyperparasitoids seem to be also able to use herbivore-induced plant volatiles changed by previous oviposition: hyperparasitism rates of *C. glomerata* cocoons were higher on plants previously infested with eggs compared to plants infested with caterpillars only. It seems that plants use eggs as a reliable information for upcoming herbivory and respond accordingly with induced defences. These defences affect the subsequently feeding larvae directly through changes in plant quality and indirectly by altering herbivore-induced plant volatiles. Parasitoids and hyperparasitoids are able to use these changes in volatile emission and successfully find and parasitise their hosts. Interestingly, plants that were exposed to egg deposition also displayed a higher number of seeds compared to plants that were not previously exposed to eggs. Thus, egg deposition could actually influence plant-associated community members from herbivores to hyperparasitoids in a food chain and benefit plant fitness. More research should be conducted regarding plant-mediated interactions due to oviposition, and how these interactions affect the structure of insect communities in nature. It will be interesting for future studies to focus on how plants recognise specific herbivore eggs and what might be the elicitors from eggs that induce the changes in plant quality. Chemical elicitors that mediate plant responses in the field and increase attraction of natural enemies are promising to be used in integrated pest management to enhance environmentally benign plant protection against pests.

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P.S. I am sorry for all the tears coming out of your eyes while reading through my acknowledgements. After all, I come from a country where drama was actually invented ☺.

Curriculum Vitae

Foteini Paschalidou was born in Athens, Greece. She received her basic education in Athens. In 2000, she moved to Kefolonia to study Organic farming with specialization in Entomology. Her BSc thesis was in collaboration with Benaki Phytopathological Institute. She investigated the effects of different diatomaceous earth formulations against the lesser grain borer on different grains. Interest in agricultural research and ecological interactions between insects and plants motivated her to pursue a Master degree at Wageningen University in The Netherlands. She carried out her Master thesis in the Laboratory of Entomology (Wageningen University) on a project entitled "Can the parasitic wasp *Trichogramma evanescens* learn to hitch-hike with mated females of its hosts, the Cabbage White butterflies *Pieris rapae* and *Pieris brassicae?"* under the supervision of Dr. Ties Huigens. After she graduated she continued working as a research assistant in the Laboratory of Entomology until 2011, involved in a project about memory consolidation in parasitic wasps and evolutionary importance regarding host location and host suitability. In 2011, she started working towards her PhD in the Laboratory of Entomology (Wageningen University) on a project on the effects of egg deposition on plant defences to insect herbivores and parasitoids. The PhD project was supervised by Prof. dr. Marcel Dicke, Prof. dr. Joop J. A. van Loon, and Dr. Nina E. Fatouros. The project was funded by N.W.O. (The Netherlands Organization for Scientific Rearch). Results of the PhD studies exploring the effects of egg deposition on plant-insect interaction are presented in this book. Since May 2015, Foteini works a post-doc research at Eidgenössische Technische Hochschule Zürich (ETH), Zurich, in the group of Biocommunication and Entomology under the supervision of Prof. dr. Consuelo de Moraes.

Publications

F. G. Pashalidou, E. Frago, E. Griese, E. H. Poelman, J. J. A. van Loon, M. Dicke, & N. E. Fatouros. 2015. Early herbivore alert matters: plant-mediated effects of egg deposition on higher trophic levels benefit plant fitness. *Ecology Letters*, in press (DOI: 10.1111/ele.12470)

F. G. Pashalidou, N. E. Fatouros, J. J. A. van Loon, M. Dicke, & R. Gols. 2015. Plant-mediated effects of butterfly egg deposition on subsequent caterpillar and larval development across different species of wild Brassicaceae. *Ecological Entomology*, 40, 444-450

F. G. Pashalidou, R. Gols, B. W. Berkhout, B. T. Weldegergis, J. J.A. van Loon, M. Dicke, & N. E. Fatouros. 2014. To be in time: egg deposition enhances plant-mediated detection of young caterpillars by parasitoids. *Oecologia*, 177, 477-486

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

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

M. Kruidhof, **F. G. Pashalidou**, N. E. Fatouros, I. A. Figueroa, L. E. M. Vet, H. M. Smid, & M. E. Huigens. 2012. Reward value determines memory consolidation in parasitic wasps. *PLoS ONE*, 7, e39615

J. A. Harvey, **F. G. Pashalidou**, R. Soler, & T. M. Bezemer. 2010. Intrinsic competition between two secondary hyperparasitoids results in temporal trophic switch. *Oikos*, 120, 226-233

F. G. Pashalidou, M. E. Huigens, M. Dicke, & N. E. Fatouros. 2010. The use of oviposition-induced plant cues by *Trichogramma* egg parasitoids. *Ecological Entomology*, 35, 748-753

M. E. Huigens, J. B. Woelke, **F. G. Pashalidou**, T. Bukovinszky, H. M. Smid, & N. E. Fatouros. 2010. Chemical espionage on species-specific butterfly anti-aphrodisiacs by hitchhiking Trichogramma wasps. *Behavioral Ecology*, 21, 470-478

N. E. Fatouros, **F. G. Pashalidou**, W. V. A. Cordero, J. J. A. van Loon, R. Mumm, M. Dicke, M. Hilker, & M. E. Huigens. 2009. Anti-aphrodisiac compounds of male butterflies increase the risk of egg parasitoid attack by inducing plant synomone production. *Journal of Chemical Ecology*, 35, 1373–1381

M. E. Huigens, **F. G. Pashalidou**, M. Qian, T. Bukovinszky, H. M. Smid, J. J. A. van Loon, M. Dicke, & N. E. Fatouros. 2009. Hitch-hiking parasitic wasp learns to exploit butterfly antiaphrodisiac. *Proceedings of the National Academy of Sciences of the U.S.A.*, 106, 820-825

N. G. Kavallieratos, C. G. Athanassiou, M. P. Michalaki, Y. A. Batta, H. A. Rigatos, **F. G. Pashalidou**, G. N. Balotis, Z. Tomanović, & B. J. Vayias. 2006. Effect of the combined use of *Metarhizium anisopliae* (Metschinkoff) Sorokin and diatomaceous earth for the control of three stored-product beetle species. *Crop Protection,* 25, 1087-1094

N. G. Kavallieratos, C. G. Athanassiou, **F. G. Pashalidou**, N. S. Andris, & Ž. Tomanović. 2005. Influence of grain type on the insecticidal efficacy of two diatomaceous earth formulations against *Rhyzopertha dominica* (F.) (Coleoptera: Bostrychidae). *Pest Management Science*, 61, 660-666.

Education Statement

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: **Foteini Paschalidou**
Date: **18 September 2015**
Group: **Laboratory of Entomology**
University: **Wageningen University & Research Centre**

1) Start-up phase	*date*
► **First presentation of your project**	
Effects of insect herbivore egg deposition on interactions of plants with their insect community: a multitrophic approach	Feb, 2011
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	

Subtotal Start-up Phase *1.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student days, University of Amsterdam	Nov 30, 2012
EPS PhD student days, Leiden University	Nov 29, 2013
► **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 UR	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**	
NERN 2012, Lunteren	Feb 07-08, 2012
NWO-ALW Plant Sciences meeting, Lunteren	Apr 02-03, 2012
NERN 2013, Lunteren	Feb 05-06, 2013
NWO-ALW Plant Sciences meeting, Lunteren	Apr 22-23, 2013
NERN 2014, Lunteren	Feb 11-12, 2014
► **Seminars (series), workshops and symposia**	
Symposium YELREM	Jun 07, 2011
6th Workshop Plant Insect Interactions, University of Amsterdam	Nov 23, 2011

	Symposium YELREM	May 30, 2012
	7th Workshop Plant Insect Interactions, Leiden University	Nov 28, 2012
	Expectation Day PhD 2013	Feb 01, 2013
	Symposium YELREM	May 17, 2013
	8th Workshop Plant Insect Interactions, Wageningen UR	Sep 24, 2013
	Symposium YELREM	May 21, 2014
	9th Workshop Plant Insect Interactions, Utrecht University	Nov 03, 2014
	Local monthy seminars at the Laboratory of Entomology	Apr 22-23, 2013
	Annual Meeting of the Netherlands Entomological Society	Feb 11-12, 2014
►	**Seminar plus**	
►	**International symposia and congresses**	
	14th International Symposium on Insect-Plant Relationships, Wageningen	Aug 13-17, 2011
	Gordon research conference on Plant-herbivore Interactions, Ventura, CA, USA	Feb 24-Mar 01, 2013
	15th International Symporium on Insect-Plant Relathionships, Switzerland	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
	6th Workshop Plant Insect Interactions (Oral)	Nov 23, 2011
	Annual Meeting of the Netherlands Entomological Society Oral)	Dec 16, 2011
	7th Workshop Plant Insect Interactions (Oral)	Nov 28, 2012
	Gordon research conference on Plant-herbivore Interactions, Ventura, CA, USA (Poster)	Feb 24-Mar 01, 2013
	Eurovol Summer School on plant volatiles (Poster)	Sep 09-12, 2013
	8th Workshop Plant Insect Interactions (Oral)	Sep 24, 2013
	Entomology PhD excursion Switzerland (Oral)	Oct 28-Nov 01, 2013
	15th International Symporium on Insect-Plant Relathionships, Switzerland (Oral)	Aug 17-22, 2014
	9th Workshop Plant Insect Interactions (Oral)	Nov 03, 2014
	British and French Ecological Societies- joint annual meeting, Lille, France (Poster)	Dec 09-12, 2014
►	**IAB interview**	
	Meeting with a member of the International Advisory Board of EPS	Sep 29, 2014
►	**Excursions**	
	Entomology PhD excursion Switzerland	Oct 28-Nov 01, 2013

Subtotal Scientific Exposure *30.1 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
6th Utrecht PhD Summer school on Environmental Signalling	Aug 22-24, 2011
Introduction to R for statistical analysis	Nov 24-25, 2011
Basic Statistics	Dec 13-15, 20-21, 2011
Mixed-Linear Models	Jun 20-21, 2012
Multivariate analysis	Oct 09-11, 18-19, 2012
► **Journal club**	
PhD journal club Entomology	2011-2015
Insect Plant Interactions discussion group at Entomology	2011-2015
► **Individual research training**	
Subtotal In-Depth Studies	*7.5 credits**

4) Personal development	*date*
► **Skill training courses**	
PhD competence assesment	May 24 & Jun 26, 2011
Project and time managment	Sep 27, Oct 12 & Nov 08, 2011
Techniques for Writing and Presenting a Scientific Paper (TWP)	Feb 14-17,2012
Effective behavior	Nov 08 & 29, 2012
Adobe InDesign	Nov 06, 2013
► **Organisation of PhD students day, course or conference**	
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.0 credits**

TOTAL NUMBER OF CREDIT POINTS*	**43.1**

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.*

Appendices

Chapter 2

Table S1. Mean (±SE) body weight (mg) of *P. brassicae* and *M. brassicae* caterpillars by different egg treatments.

8 d after oviposition				
	(Eggs(+)/HR+) (mean±SE)	Eggs(-) (mean±SE)	(Eggs(+)/HR-) (mean±SE)	Eggs(-) (mean±SE)
Conspecific caterpillars (mg)				
P. brassicae eggs	1.04 ±0.02	1.37±0.01	1.24 ± 0.02	1.31± 0.01
M. brassicae eggs	0.67 ± 0.01	0.69 ± 0.01		
Heterospecific caterpillars (mg)				
P. brassicae eggs	0.40 ± 0.01	0.61 ± 0.01	0.38 ± 0.01	1.56± 0.01
M. brassicae eggs	1.17 ± 0.02	1.20±0.01		
12 d after oviposition				
Conspecific caterpillars (mg)				
P. brassicae eggs	14.77 ± 0.7	23.39±0.8	20.26 ± 0.8	24.68 ± 0.8
M. brassicae eggs	4.5 ± 0.21	4.73 ± 0.23		
Heterospecific caterpillars (mg)				
P. brassicae eggs	1.61 ± 0.07	2.86 ± 0.13	1.76 ± 0.08	2.92 ± 0.1
M. brassicae eggs	17.74 ± 0.9	18.47 ± 0.9		

Table S2. Mean (±SE) body weight (mg) of *P. brassicae* and *M. brassicae* caterpillars, when all excess eggs were removed after oviposition (additional control experiment).

8 d after oviposition		
	Eggs(+) (mean±SE)	Eggs(-) (mean±SE)
P. brassicae eggs	1.94±0.05	1.91±0.06
M. brassicae eggs	0.58 ± 0.01	0.61 ± 0.01
12 d after oviposition		
P. brassicae eggs	26.56 ± 0.57	26.43±0.52
M. brassicae eggs	0.93± 0.021	0.99 ± 0.02

Table S3. General linear model for estimating the effect of eggs and caterpillars, on plant growth between 5 to 12 days after oviposition.

Source	_df_	_df_ resid	resid. dev	_F_	_P_
Basis			30759		
Eggs (E)	2	113	22016	22.852	0.001***
Caterpillars (C)	1	112	21454	2.935	0.089
E x C	2	110	21043	1.074	0.345

Coefficients

		Estimate	Std. Error	t value	_P_
Intercept	β_0	31.466	2.525	12.461	0.001***
Egg _M_	β_1	-6.266	5.050	-1.241	0.217
Egg _P_	β_2	12.333	3.993	3.089	0.003 **
Caterpillar _P_	β_3	2.354	3.634	0.648	0.518
Egg _M_*Cater _P_	β_4	2.054	7.174	0.286	0.775
Egg _P_*Cater _P_	β_5	7.345	5.779	1.271	0.206

Terms added sequentially (first to last), M: Mamestra brassicae, P: Pieris brassicae

Table S4. General linear model for estimating the effect of eggs on plant developmental phase 5 days after oviposition.

Coefficients

	Estimate	Std. Error	z value	_P_
Intercept	-0.904	0.2875	-3.146	0.002 **
Egg _M_	0.131	0.5712	0.230	0.818
Egg _P_	1.443	0.4424	3.263	0.001 **

M: Mamestra brassicae, P: Pieris brassicae

Chapter 3

Table S1. Results from the mixed models testing the effects of egg deposition on larval biomass of the herbivore *P. brassicae* on different Brassicaceae species. Egg treatment on *M. moricandioides* is included as the reference category (intercept). The output of the LMM fitted with REML is presented in terms of estimates, standard error (SE), t value, and P value.

	Estimate	SE	*t* value	P value
Intercept	3.63	0.49	7.311	<0.001
treatment	-0.1	0.2	-0.52	0.599
species_*B. nigra*	2.8	0.59	4.69	<0.001
species_*B. oleracea*	-0.03	0.59	4.69	0.95
species_*M. arvensis*	2.7	0.68	3.96	0.001
species_*S. arvensis*	2.39	0.59	4.01	0.001
egg**B. nigra*	-0.26	0.22	-1.18	0.23
egg**B. oleracea*	-0.33	0.22	-1.48	0.13
egg**M. arvensis*	-1.07	0.24	-4.33	<0.001
egg**S. arvensis*	-0.05	0.22	-0.25	0.8

Table S2. Results from the linear mixed model (LMM) statistical analysis testing the effects of *P. brassicae* egg deposition on the caterpillar mass of the individuals hatching from these eggs for each of the plant species. *Pieris brassicae* developed on plants previously infested with eggs (eggs(+)) or on egg-free plants (eggs(-)). Eggs(-) plants is included as a reference category (intercept) and eggs(+) plants as treatment. The output of the LMM fitted with REML is presented in terms of estimates (Est), standard error (SE), t value, F and P value and n represents number of replicates.

		Intercept			Treatment			F value	P value
Species	n	Est	SE	*t* value	Est	SE	*t* value		
B. nigra	15	6.43	0.19	33.42	-0.37	0.09	-4.18	17.46	<0.001
B. oleracea	15	3.59	0.60	6.03	-0.44	0.08	-5.42	29.36	<0.001
M. arvensis	8	3.41	0.27	12.60	-0.11	0.19	-0.56	0.31	0.58
M. moricandioides	15	6.33	0.28	22.33	-1.18	0.14	-8.22	67.64	<0.001
S. arvensis	15	6.05	0.26	23.46	-0.16	0.09	-1.88	3.54	0.06.

Table S3. Results from the linear mixed model (LMM) statistical analysis testing the effects of *P. brassicae* egg deposition on pupal mass of the individuals hatching from these eggs for each of the plant species. *Pieris brassicae* developed on plants previously infested with eggs (eggs(+)) or on egg-free plants (eggs(-)). Eggs(-) plants is included as a reference category (intercept) and eggs(+) plants as treatment. The output of the LMM fitted with REML is presented in terms of estimates (Est), standard error (SE), t value, F and P value and n represents number of replicates.

		Intercept			Treatment			F value	P value
Species	n	Est	SE	*t* value	Est	SE	*t* value		
B. nigra	15	415.21	11.22	37.01	-28.74	7.66	-3.75	14.09	<0.001
B. oleracea	15	304.37	8.65	35.19	-22.04	5.27	-4.18	17.50	<0.001
M. arvensis	8	361.74	19.03	19.01	0.61	11.37	0.05	0.00	0.96
M. moricandioides	15	346.74	15.11	22.96	4.37	10.81	0.40	0.16	0.69
S. arvensis	15	424.51	7.68	55.26	-20.38	5.87	-3.47	12.05	<0.001

Table S4. Results from the linear mixed model (LMM) statistical analysis testing the effects of *P. brassicae* egg deposition on developing time of individuals hatching from these eggs for each of the plant species. *Pieris brassicae* developed on plants previously infested with eggs (eggs(+)) or on egg-free plants (eggs(-)). Eggs(-) plants is included as a reference category (intercept) and eggs(+) plants as treatment. The output of the LMM fitted with REML is presented in terms of estimates (Est), standard error (SE), t value, F and P value and n represents number of replicates.

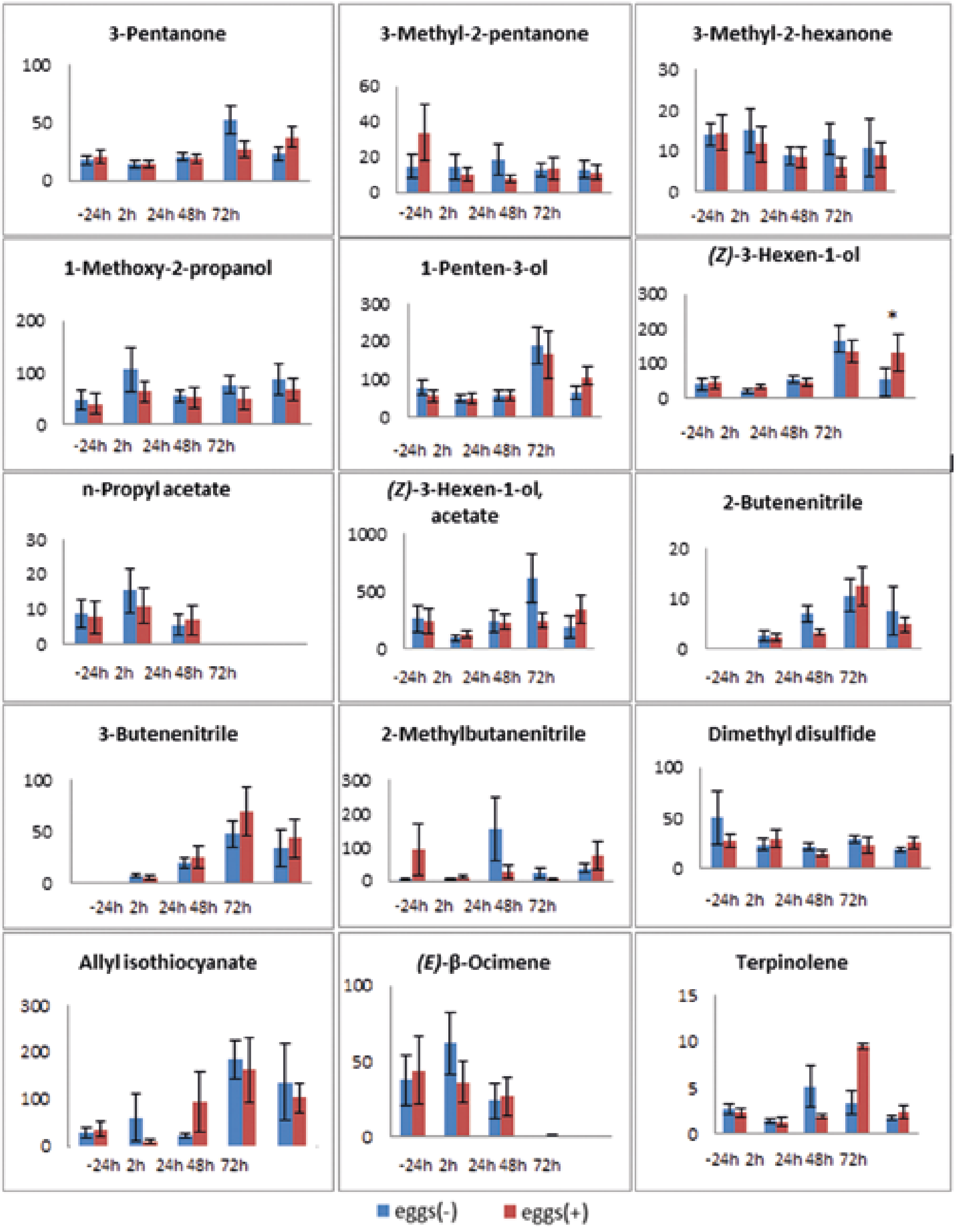
		Intercept			Treatment			F value	P value
Species	n	Est	SE	*t* value	Est	SE	*t* value		
B. nigra	15	29.00	0.45	64.67	0.78	0.19	3.98	15.88	<0.001
B. oleracea	15	33.13	0.48	69.35	1.22	0.30	3.98	15.87	<0.001
M. arvensis	8	33.68	0.60	55.99	0.62	0.54	1.13	1.27	0.26
M. moricandioides	15	33.96	0.52	64.85	0.74	0.37	1.98	3.92	0.048
S. arvensis	15	28.967	0.35	82.41	0.395	0.17	2.32	5.38	0.020

Chapter 4

Methods S1

Analytical conditions- Experimental Protocol for plant volatile separation and detection

Thermo Trace GC Ultra in combination with Thermo Trace DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA) were used for separation and detection of plant volatiles. Prior to release of the volatiles, each sample was dry-purged under a stream of nitrogen (50 ml min^{-1}) for 10 min at ambient temperature in order to remove moistures. The collected volatiles were then released from the Tenax TA thermally on an Ultra 50:50 thermal desorption unit (Markes, Llantrisant, UK) at 250 ^{o}C for 10 min under helium flow of 20 ml min^{-1}, while re-collecting the volatiles in a thermally cooled universal solvent trap: Unity (Markes) at 10 ^{o}C. Once the desorption process was completed, volatile compounds were released from the cold trap by ballistic heating at 40 ^{o}C s^{-1} to 280 °C, which was then kept for 10 min, while the volatiles being transferred to a ZB-5MSi analytical column [30 m x 0.25 mm I.D. x 1.00 m F.T. (Phenomenex, Torrance, CA, USA)], in a splitless mode for further separation. The GC oven temperature was initially held at 40 °C and was immediately raised at 5 ^{o}C min-1 to a final temperature of 280 °C, where it was kept for 7 min under a helium flow of 1 ml min^{-1} in a constant flow mode. The DSQ mass spectrometer (MS) was operated in a scan mode with a mass range of 35 – 400 amu at 4.70 scans s^{-1} and spectra were recorded in electron impact ionisation (EI) mode at 70 eV. The MS transfer line and ion source were set at 275 and 250 ˚C, respectively. Compound identification was based on comparison of mass spectra with those in the NIST 2005 and Wageningen Mass Spectral Database of Natural Products MS libraries. Experimentally obtained linear retention indices (LRI) were also used as additional measures for confirming the identity of compounds.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50
0
-24h 2h 24h 48h 72h
3-Methyl-2-pentanone
60
40
20
0
-24h 2h 24h 48h 72h
3-Methyl-2-hexanone
30
20
10
0
-24h 2h 24h 48h 72h
1-Methoxy-2-propanol
200
100
0
-24h 2h 24h 48h 72h
1-Penten-3-ol
300
200
100
0
-24h 2h 24h 48h 72h
(Z)-3-Hexen-1-ol
300
200
100
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-24h 2h 24h 48h 72h
n-Propyl acetate
30
20
10
0
-24h 2h 24h 48h 72h
(Z)-3-Hexen-1-ol, acetate
1000
500
0
-24h 2h 24h 48h 72h
2-Butenenitrile
20
10
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-24h 2h 24h 48h 72h
3-Butenenitrile
100
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0
-24h 2h 24h 48h 72h
2-Methylbutanenitrile
300
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100
0
-24h 2h 24h 48h 72h
Dimethyl disulfide
100
50
0
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Allyl isothiocyanate
300
200
100
0
-24h 2h 24h 48h 72h
(E)-β-Ocimene
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Terpinolene
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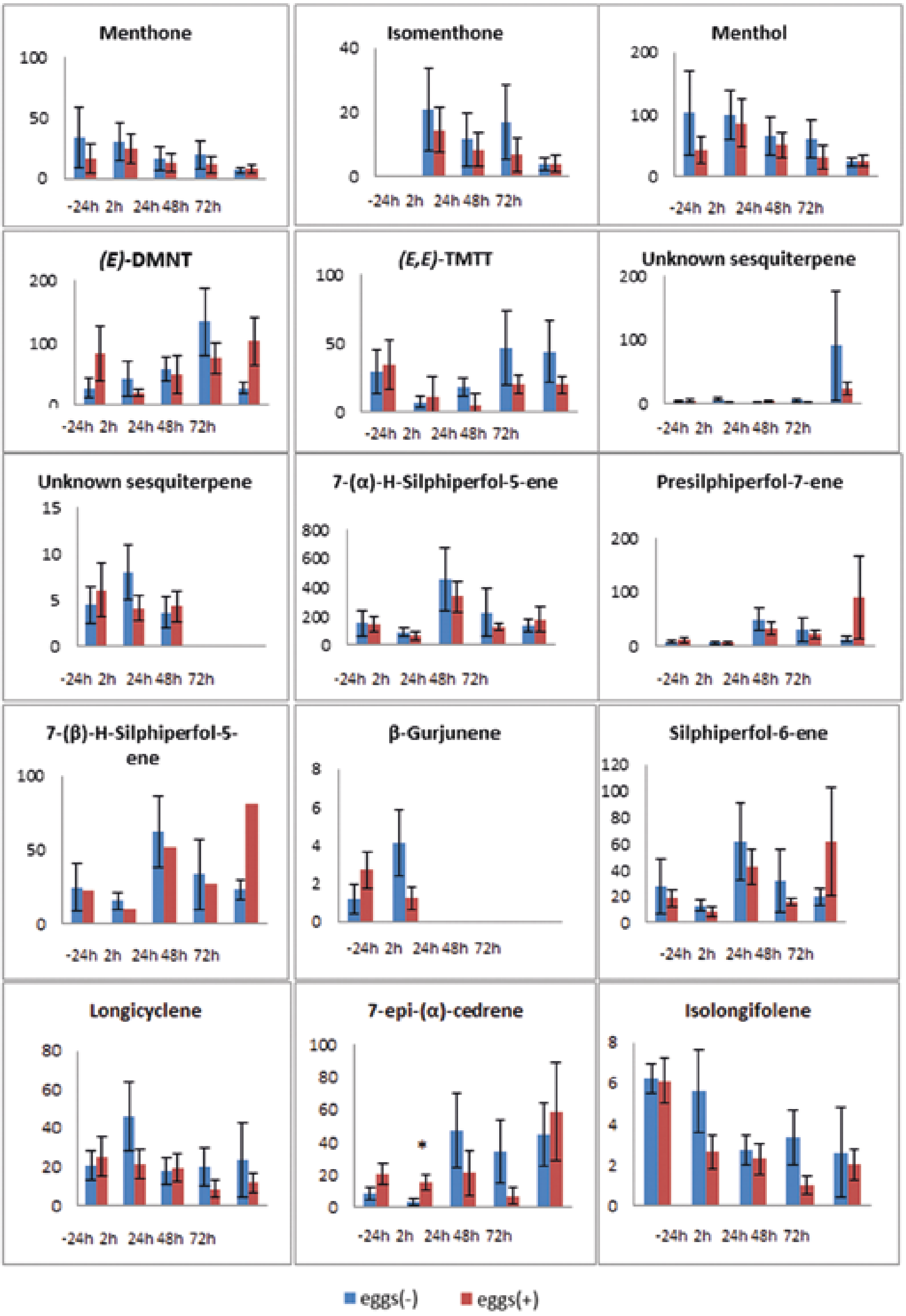
eggs(-)
eggs(+)


Menthone
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Isomenthone
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Menthol
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-24h 2h 24h 48h 72h
(E)-DMNT
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Unknown sesquiterpene
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7-(α)-H-Silphiperfol-5-ene
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-24h 2h 24h 48h 72h
Presilphiperfol-7-ene
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7-(β)-H-Silphiperfol-5-ene
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β-Gurjunene
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6
4
2
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Silphiperfol-6-ene
120
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-24h 2h 24h 48h 72h
Longicyclene
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7-epi-(α)-cedrene
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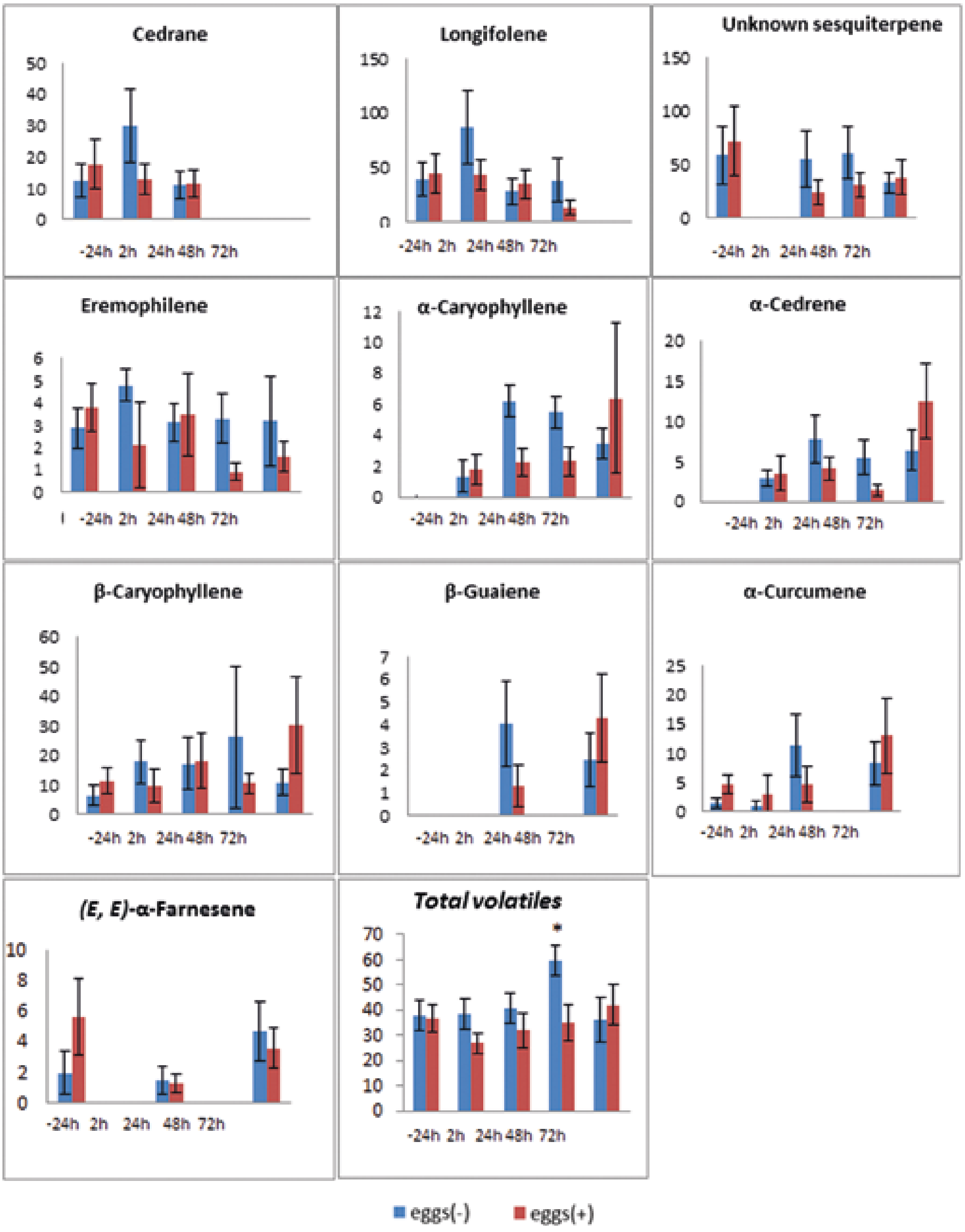


Figure S1. Volatile emission by eggs (+) plants with (2 h,24 h, 48 h, 72 h) or without *Pieris brassicae* caterpillars (-24 h) compared to eggs(-) plants with or without caterpillars, at various time points. Volatile emissions are given as mean peak area ± SE/g fresh weight/ 10^{-4}. Number of plants tested per treatment per time point = 9 for (-24 h), 10 for the rest of the time points. Asterisks indicate significant differences within the time point *= *P* <0.05 (Mann-Whitney *U*-test)

Table S1. Volatile emissions[1] by *Brassica nigra* plants: uninfested (C), infested only with *Pieris brassicae* eggs 24 h before hatching (E), infested with eggs and caterpillars 2 h after hatching (EP2), caterpillars 2 h after hatching (P2), with eggs and caterpillars 24 h after hatching (EP24), caterpillars 24 h after hatching (P24),with eggs and caterpillars 48 h after hatching (EP48), caterpillars 48 h after hatching (P48), with eggs and caterpillars 72 h after hatching (EP72), caterpillars 72 h after hatching (P72h).

	Treatment →	C	E	EP2	P2	EP24	P24	EP48	P48	EP72	P72
ID [2]	Volatile compounds↓	(N=9)	(N=9)	(N=10)	(N=10)	(N=10)	(N=10)	(N=10)	(N=10)	(N=10)	(N=10)
	Ketones										
5	3-Pentanone	20±5	18±4	15±3	14±3	19±3	21±4	27±7	52±12	38±9	23±6
9	3-Methyl-2-pentanone	34±16	15±7	10±4	14±7	8±2	19±9	14±6	12±4	11±4	13±5
10	3-Methyl-2-hexanone	14±4	14±3	12±4	15±5	9±3	9±2	6±2	13±4	9±3	11±7
	Alcohols										
3	1-Methoxy-2-propanol	40±18	47±20	63±21	106±42	52±9	55±11	51±8	76±17	67±21	87±31
4	1-Penten-3-ol	56±15	77±19	50±14	51±10	58±14	57±14	165±61	189±47	104±29	64±18
11	(*Z*)-3-Hexen-1-ol	43±18	41±16	31±7	21±4	45±10	52±12	132±32	162±46	**130±52**^{a}	**54±32**^{b}
	Esters										
6	n-Propyl acetate	8±5	9±4	11±5	15±6	7±4	5±3	-	-	-	-
13	(*Z*)-3-Hexen-1-ol, acetate	236±109	259±114	119±33	92±28	228±66	240±96	243±69	612±209	338±119	185±98
	N and/or S containing compounds										
1	2-Butenenitrile	-	-	2±1	3±1	3±1	7±2	12±4	11±3	5±1	8±5
2	3-Butenenitrile	-	-	6±2	8±2	25±11	20±5	69±23	48±13	43±18	35±18
7	2-Methylbutanenitrile	92±77	5±3	10±3	6±2	27±22	154±96	5±2	22±17	76±41	37±14

8	Dimethyl disulfide	28±6	50±27	29±9	24±6	15±3	22±4	23±7	29±4	26±6	19±3
12	Allyl isothiocyanate	36±17	28±11	10±4	60±50	94±64	22±6	162±68	184±41	103±32	136±82
	Monoterpenes										
14	(*E*)-β –Ocimene	44±23	37±17	36±13	62±20	27±13	24±12	-	-	-	-
15	Terpinolene	2±1	3±1	1±1	1.3±0.2	1.8±0.4	5±2	1.2±0.3	3±1	2±1	1.7±0.3
17	Menthone	16±12	33±25	24±12	30±16	13±7	17±10	11±7	19±11	8±3	7±3
18	Isomenthone	-	-	14±7	21±13	8±5	11±8	7±5	17±11	4±3	4±2
19	Menthol	43±21	102±68	85±38	98±40	50±20	65±31	32±19	61±31	25±10	24±7
	Homoterpenes										
16	(*E*)-DMNT[3]	83±45	27±15	20±5	42±29	49±30	59±19	75±24	134±54	102±38	27±10
40	(*E*,*E*)-TMTT[4]	34±17	30±16	11±15	7±4	5±9	18±7	20±7	47±27	20±7	44±23
	Sesquiterpenes										
20	Unknown sesquiterpene	54±21	90±43	122±70	218±103	46±18	69±33	-	-	-	-
21	Unknown sesquiterpene	31±12	32±12	38±15	73±28	18±6	23±8	10±5	27±12	11±4	34±32
22	7-(α)-H-Silphiperfol-5-ene	147±52	151±90	65±27	91±28	335±106	457±217	126±27	227±169	179±91	136±44
23	Presilphiperfol-7-ene	12±6	8±3	6±3	6±2	34±11	49±21	21±7	29±22	89±77	12±5
24	7-(β)-H-Silphiperfol-5-ene	23±9	25±16	10±4	16±5	52±15	62±24	27±6	34±23	81±57	23±7
25	β-Gurjunene	3±1	1±1	1±1	4±2	-	-	-	-	-	-
26	Silphiperfol-6-ene	19±6	28±21	8±3	13±5	42±13	62±29	16±3	32±24	62±41	20±6

27	Longicyclene	25±10	21±8	21±8	46±18	19±7	18±7	8±4	20±10	11±5	23±19
28	7-epi-(α)-Cedrene	20±7	9±4	**15±5**^{a}	**4±2**^{b}	21±14	47±23	7±5	34±20	59±30	45±19
29	Isolongifolene	6±1	6±1	3±1	6±2	2±1	3±1	1±0	3±1	2±1	3±2
30	Cedrane	18±8	12±5	13±5	30±12	12±5	11±4	-	-	-	-
31	Longifolene	44±18	39±15	43±14	86±34	35±13	28±12	13±7	38±20	-	-
32	Unknown sesquiterpene	72±33	58±27	-	-	24±12	55±26	30±12	61±24	38±16	33±10
33	Eremophilene	4±1	3±1	2±2	5±1	3±2	3±1	1±0	3±1	2±1	3±2
34	α-Caryophyllene	-	-	2±1	1±1	2±1	6±3	2±1	5±2	6±5	3±2
35	α-Cedrene	-	-	3±2	3±1	4±1	8±3	1±1	5±2	13±5	6±3
36	β-Caryophyllene	11±4	6±3	10±6	18±7	18±9	17±9	11±3	26±24	30±16	11±4
37	β-Guaiene	-	-	-	-	1±1	4±2	-	-	4±2	2±1
38	α-Curcumene	48±23	42±19	-	-	17±8	38±17	21±8	42±18	25±11	24±7
39	(*E*,*E*)-α-Farnesene	6±2	2±1	-	-	1±1	1±1	-	-	4±1	5±2
	average volatile emission	38±6	37±6	27±4	39±6	32±7	41±9	**35±7**^{a}	**60±14**^{b}	36±5	42±8
	N of increased compounds per time point	18	14	**7**^{1}	**25**^{2}	**9**^{1}	**27**^{2}	**3**^{1}	**30**^{2}	23	11

[1] Volatile emissions are given as mean peak area ±SE/g fresh weight of foliage divided by 10^4 with the number of samples between brackets

[2] ID corresponds with the numbers presented in Fig. 2

[3] (*E*)-DMNT= 4,8-Dimethylnona-1,3,7-triene

[a, b] Significant differences ($P<0.05$) between treatments (Mann-Whitney-*U*-test)

[4] (*E*,*E*)-TMTT=4,8,12-Trimethyl-1,3,7,11-tridecatetraene

[1, 2] Significant differences ($P<0.05$) between treatments (sign-test)

Table S2. Percentage of first landings of *Cotesia glomerata* female wasps on egg-infested (24h before hatching, (-24h)) eggs(+) and caterpillar-infested (1-48 after hatching) eggs(-) plants (test) against clean *B. nigra* plants (control) in a windtunnel set-up. Eight biological replicates were conducted per time point and at least n = 6-15 wasps were tested per plant pair. Asterisks indicate a preference which is significantly different from a 50:50 distribution within a choice test, * P< 0.05, *** P < 0.001, (GLM).

Time (h)	% of first landings test	% of first landings control	P values
-24	76	24	0.007*
1	45	55	0.36
2	46	54	0.43
8	65	35	0.03*
24[1]	70	30	< 0.001***
48	82	18	< 0.001***

[1] Ponzio, C., Gols, R., Weldegergis, B.T. & Dicke, M. (2014). Caterpillar-induced plant volatiles remain a reliable signal for foraging wasps during dual attack with a plant pathogen or non-host insect herbivore. *Plant, Cell and Environment*, 37, 1924-1938

Chapter 5

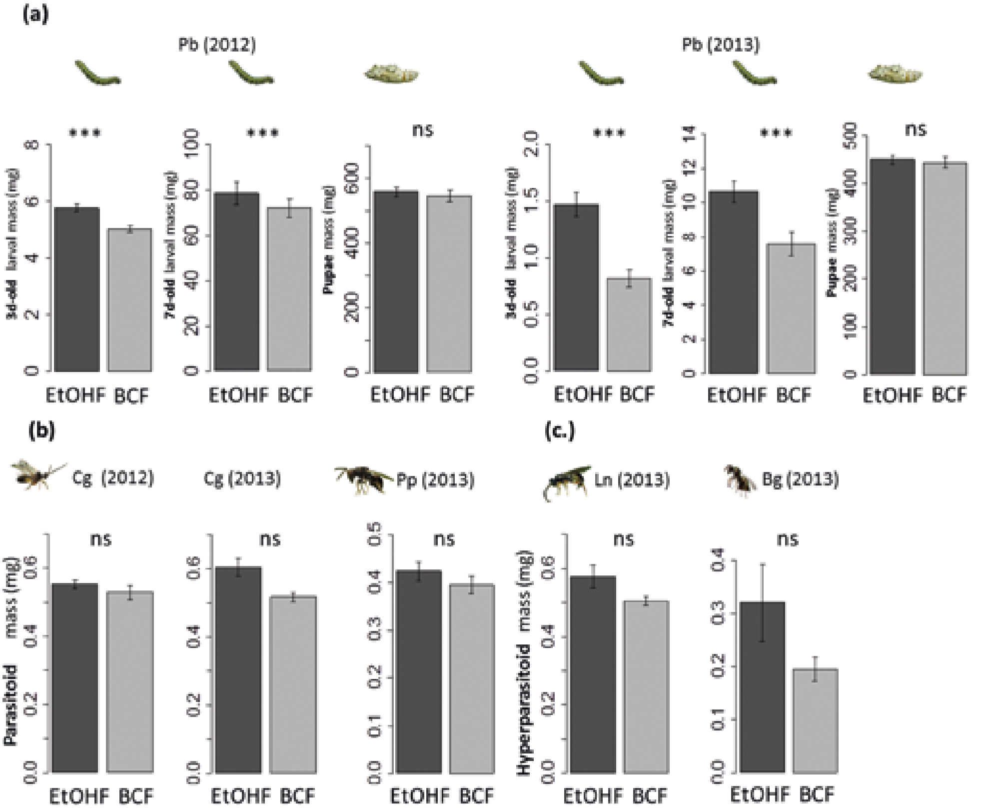


Figure S1. Plant-mediated effects of chemical elicitor benzyl cyanide (BC) treatment on caterpillar and pupal performance under field conditions for two sequential years (2012, 2013). Bars indicate body mass (mean ± SE) of (a) *P. brassicae* (Pb) caterpillars and pupae that developed on *Brassica nigra* plants and infested with caterpillars, previously treated with benzyl cyanide (BCF), or on plants threated with the solvent ethanol (EtOHF); (b) *Cotesia glomerata* (Cg) larval parasitoids and *Pteromalus puparum* (Pp) pupal parasitoids that developed on caterpillars or pupae in *B. nigra* plants: either on BC or EtOH plants; and (c) *Lysibia nana* (Ln) and *Baryscapus galactopus* (Bg) hyperparasitoids that developed in *C. glomerata* on BCF and EtOHF plants. n plants = 60. Asterisks indicate significant differences in mass among the different treatments, *** $P < 0.001$, ns: not significant, (LMM).

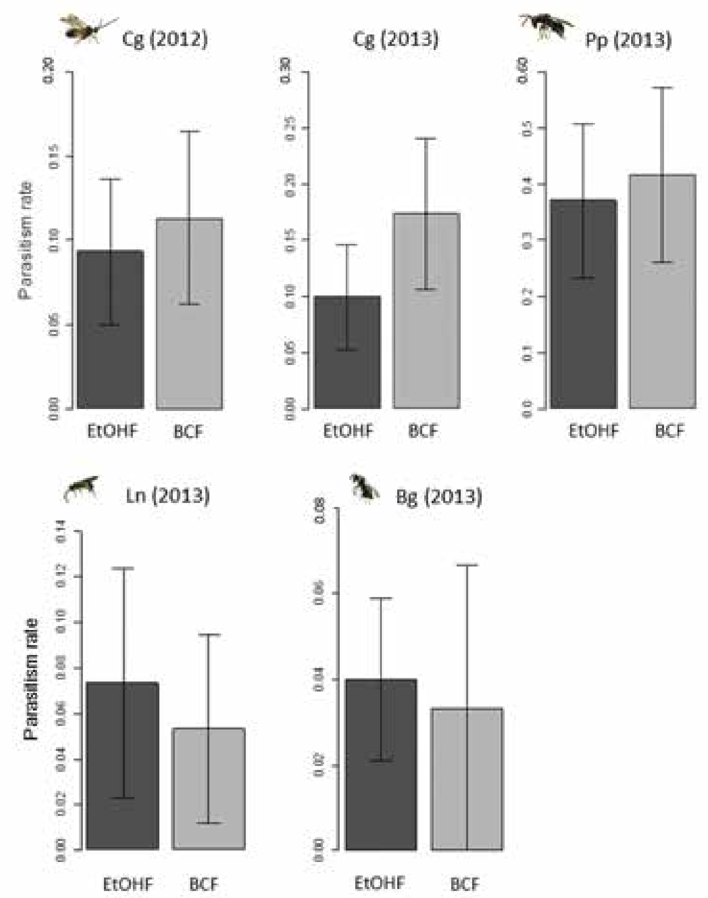
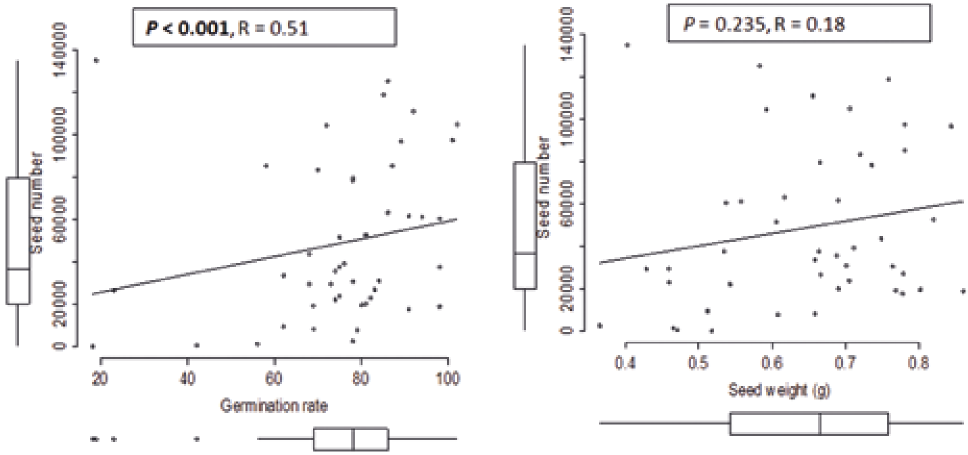


Figure S2. Plant-mediated effects of chemical elicitor benzyl cyanide treatment (BC) on parasitism rates under field conditions, in two sequential years (2012, 2013). Bars indicate parasitism rates (mean ± SE) of *Cotesia glomerata* larval and *Pteromalus puparum* pupal parasitoids that developed in *Pieris brassicae* caterpillars or pupae respectively, *Baryscapus galactopus* and *Lysibia nana* hyperparasitoids that developed in *C. glomerata* larvae or cocoons respectively, on *B. nigra* BCF (Benzyl–cyanide treated and caterpillar feeding) and EtOHF (ethanol treated and caterpillar feeding) plants. n plants = 60. (LMM).



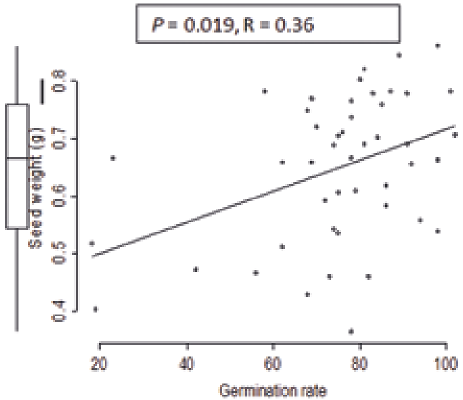


Figure S3: Pairwise correlations among *Brassica nigra* plant reproductive traits in experiments with plants exposed to *Pieris brassicae* egg deposition. Each graph shows results of a linear regression test where significant results are depicted in bold ($P < 0.016$ after Bonferroni correction). The height of the boxes represents the first to the third quartile of the range; the horizontal line within the box is the median; the whiskers indicate the data minimum and maximum.

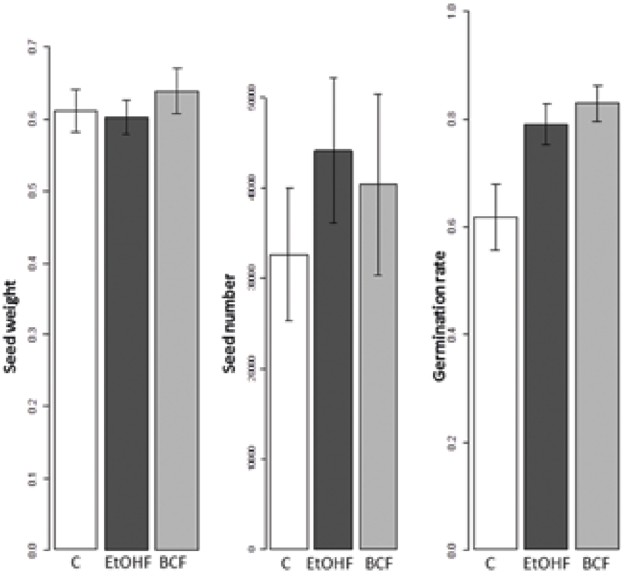


Figure S4. Plant-mediated effects of chemical elicitor benzyl cyanide treatment on *Brassica nigra* seed number, seed weight and germination rate. Plants that were infested with caterpillars were previously treated with benzyl cyanide (BCF), or the solvent ethanol (EtOHF) or left uninfested (C). Bars indicate mean ± SE of total seed weight (g), total number of seeds, germination rate, n plants = 90, (GMM).

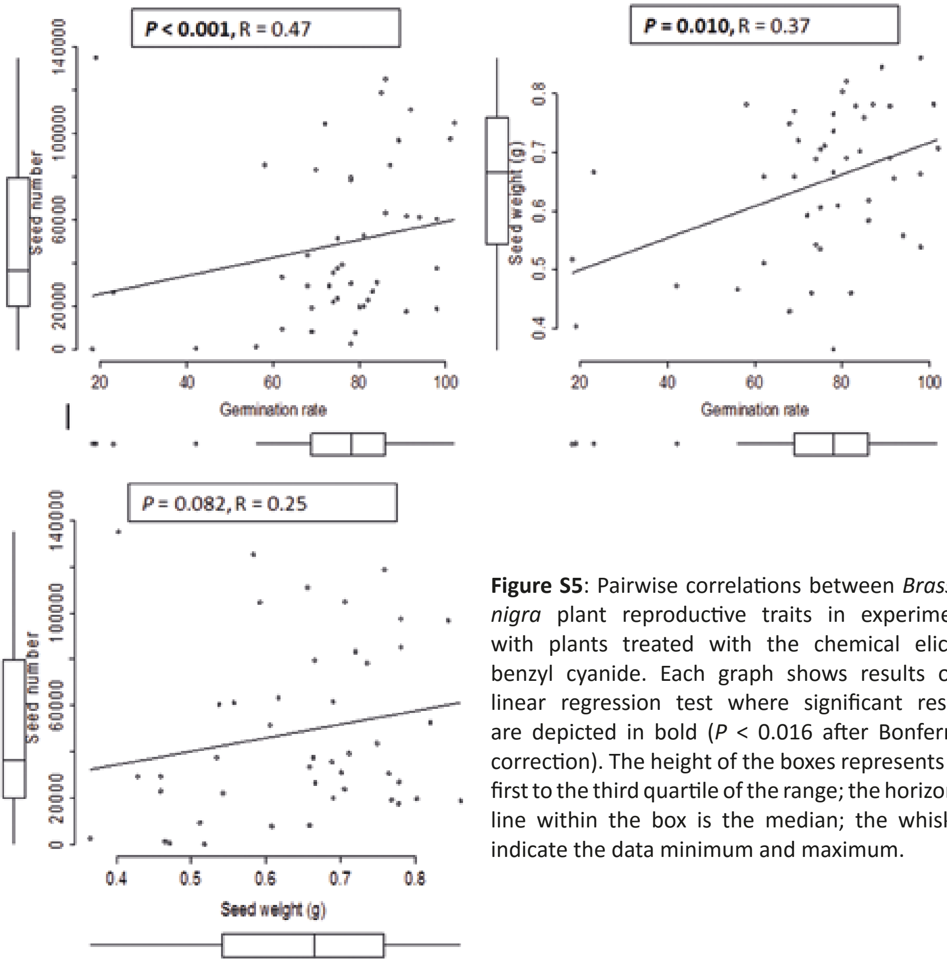


Figure S5: Pairwise correlations between *Brassica nigra* plant reproductive traits in experiments with plants treated with the chemical elicitor benzyl cyanide. Each graph shows results of a linear regression test where significant results are depicted in bold ($P < 0.016$ after Bonferroni correction). The height of the boxes represents the first to the third quartile of the range; the horizontal line within the box is the median; the whiskers indicate the data minimum and maximum.

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