Host location by hyperparasitoids:
an ecogenomic approach

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Thesis

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To my beloved parents

谨以此书，献给我最亲爱的父母

Abstract

It is fascinating that our ecological systems are structured by both direct and indirect species interactions. In terrestrial ecosystems, plants interact with many species of insects that include both harmful herbivores and beneficial natural enemies of herbivores. During the last 30 years, substantial progress has been made in different plant-insect systems regarding plant trait-mediated species interactions in a tritrophic context. However, plant-based food webs generally consist of more than three trophic levels. For example, hyperparasitoids are parasitic wasps at the fourth trophic level within the plant-associated insect community. They parasitize larvae or pupae of primary parasitoids that are broadly used in biological pest control programmes. Surprisingly, the cues that hyperparasitoids use for host location have remained largely unknown.

The studies presented in this thesis aimed to investigate the cues that are used by hyperparasitoids in host location using an ecogenomic approach that combines metabolomic, transcriptomic and proteomic tools with behavioural studies and field experiments. In addition, we addressed the role of herbivore-associated organisms in plant-mediated indirect species interactions. A naturally existing study system of the *Brassica oleracea* plant-based food web, including four trophic levels was used. In this system, the two herbivorous insect species, *Pieris brassicae* and *P. rapae,* are specialists on *Brassica* plants. The plants emit herbivore-induced plant volatiles (HIPVs) in response to *Pieris* caterpillar feeding damage which results in attraction of natural enemies of the herbivores, i.e. *Cotesia* wasps. These parasitic wasps, in turn, are attacked by hyperparasitoids, such as *Lysiba nana*. The results presented in this thesis show that hyperparasitoids also use HIPVs for host searching. Interestingly, they are especially attracted by plant odours induced by parasitized caterpillars. Moreover, hyperparasitoids can also use caterpillar body odours to find their hosts at close distance. These findings indicate that infochemicals are the major cues that mediate host searching behaviour of hyperparastioids. Similar to other herbivore-associated organisms, parasitoid larvae feeding inside a herbivore host can induce both behavioral and physiological changes in the host. To further investigate how parasitoid larvae indirectly affect plant responses to herbivory and plant volatile-mediated multitrophic interactions, the role of caterpillar labial salivary glands in plant-hyperparasitoid interactions were investigated. The secretions of labial saliva were eliminated by using an ablation technique. Remarkably, the results show that when the labial salivary glands of the caterpillars were completely removed, plants induced by either unparasitized or *Cotesia glomerata*-parasitized caterpillars were equally attractive to the hyperparasitoid. Moreover, plants became less attractive to the hyperparasitoid when damaged by ablated caterpillars compared to plants damaged by mock-treated caterpillars and the hyperparasitoids were not able to distinguish between volatiles emitted by herbivore-damaged plants and undamaged control plants when caterpillar salivary glands had been removed. These results suggest that parasitism alters the composition of labial saliva of parasitized caterpillar, which thereby alters the plant phenotype and subsequently plant-hyperparasitoid interactions. The outcomes of this thesis contribute to our understanding of the role of infochemicals in foraging decisions of hyperparasitoids.

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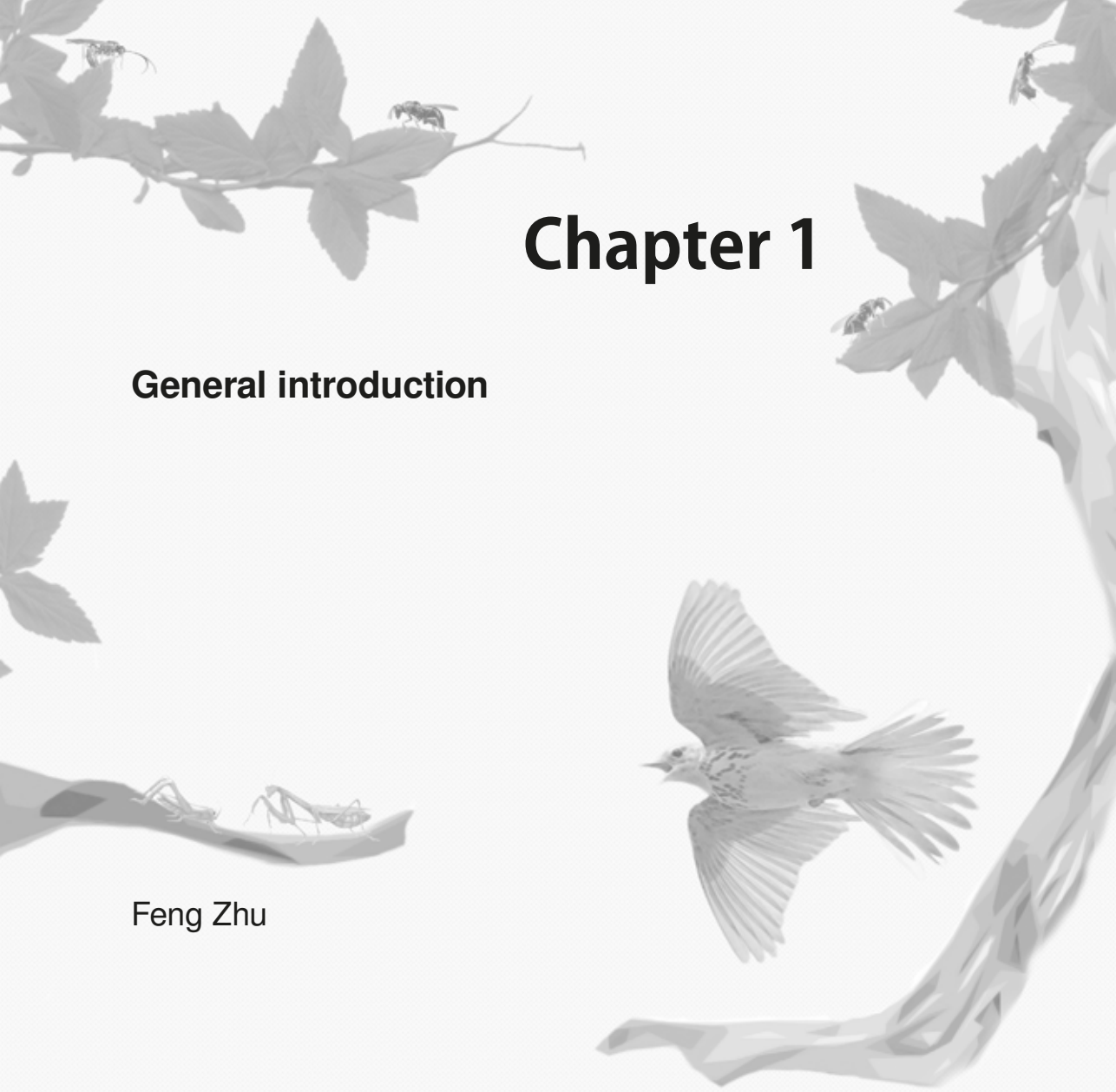
Chapter 1

General introduction



Feng Zhu

Ecological studies have extensively demonstrated the complexity of species interactions in communities that range from direct trophic relationships to intricate indirect interaction networks (Polis & Strong 1996). One of the most famous examples of food chains and their indirect interactions was reported in *The Origin of Species* by Darwin (1859), involving bumble bees that pollinate red clover; though some bees may be eaten by field mice, in turn, the mice may be attacked by domestic cats. Thereafter, Darwin made a remarkable speculation that if the cats were removed from this food chain, the red clover plants would eventually remain unpollinated, because the mice would eliminate the bees. The major components of a food chain in terrestrial ecosystem are primary producers (such as plants), consumers (herbivores), intermediate-level predators and top predators. The relationships and interactions between organisms at the same or different trophic levels significantly affect the structure of food webs, as well as population dynamics. Thus far, people have extended their observations and predictions on species interactions in food webs in different types of ecosystems, attempting to explain possible similarities and differences among them (Chase 2000).

In terrestrial ecosystems, plants, besides struggling for survival under various abiotic stresses, are constantly challenged by herbivorous organisms because they are the primary sources of energy for this second trophic level. One of the important groups of herbivores on plants are herbivorous insects. In order to defend themselves against attack by herbivorous insects, plants have evolved a suite of constitutive and induced defence mechanisms (Mithofer & Boland 2012). On the one hand, constitutive defences are generally “static” plant traits and act as physical barrier (wax layer or lignification in plant tissue), or stored plant toxins that act as feeding or oviposition deterrents or can intoxicate feeding herbivores (Gatehouse 2002; Wittstock & Gershenzon 2002). On the other hand, induced defence mechanisms become “active” upon tissue damage by attackers; for example the production of defensive compounds can be initiated in response to herbivory (Gatehouse 2002).

Price *et al.* (1980) pointed out that plant-herbivore interactions cannot be studied realistically without consideration of natural enemies of herbivores at the third trophic level, because it is essential to understand the role of natural enemies in plant-herbivore interactions, as well as the role of plants in predator-prey relationships. Soon after, the importance of plant infochemicals (Dicke & Sabelis 1988b) in plant-herbivore-carnivore interactions had been acknowledged and further developed into plant indirect defence theory (Vet & Dicke 1992; Heil 2008). In response to herbivory, plants actively produce so-called herbivore-induced plant volatiles (HIPVs) that have been demonstrated to be used by natural enemies of herbivores for host location (Dicke & Sabelis 1988a; Godfray 1994; Agelopoulos *et al.* 1995; Turlings *et al.* 2012). Thus, recruiting natural enemies of herbivores by HIPVs may benefit plants by top-

down control of their herbivorous attackers. Besides natural enemies of herbivores, however, increasing evidence indicates that a wide range of other members of the plant-associated community (including antagonists) use HIPVs in their foraging decisions (De Moraes *et al.* 2001; Runyon *et al.* 2006; Dicke & Baldwin 2010; Karban *et al.* 2014). Thereby, plant volatiles become "public" cues and make a plant apparent to all other community members (Dicke & Baldwin 2010; Heil & Karban 2010).

It has been long recognized that natural food webs generally contain more than three trophic levels (Sullivan 1987). However, the organisms at the fourth trophic level, for example hyperparasitoids that are parasitoids attacking other parasitoids, have not drawn much attention in studies regarding plant-insect interactions. Although some studies have addressed questions on preference and performance of hyperparasitoid (Buitenhuis *et al.* 2004; Buitenhuis *et al.* 2005; Harvey 2008), there is still important lack of knowledge on their foraging behaviour and interactions with other community members.

The main objective of this thesis project was to study plant-volatile-mediated interactions in food webs up to the fourth trophic level using an ecogenomic approach that combines metabolomic, transcriptomic and proteomic tools with behavioural studies and field experiments. In this thesis, I investigated the volatile cues used by hyperparasitoids during host location. In addition, I addressed how the hosts of hyperparasitoids, parasitoid larvae feeding inside the herbivore, give away their presence by affecting host physiology and plant responses to herbivory indirectly.

Study system

To address the objectives in this thesis, a naturally existing *Brassica oleracea* plant based food web including four trophic levels was used (Figure 1).

Plant species

Brassica oleracea L. (*Brassica*ceae) is both an economically and ecologically important plant species. The cultivated forms of *B. oleracea* include several crops for common consumption, like cabbage, Brussels sprouts, cauliflower, broccoli, etc. Under natural conditions, *brassica*ceous plants host a complex insect community, including both generalist and specialist herbivorous insects, as well as carnivorous insects at higher trophic levels (Bukovinszky *et al.* 2008; Poelman *et al.* 2013).

The *brassica*ceous plants are often used for studying induced direct and indirect responses to herbivore attack (Broekgaarden *et al.* 2007; Zheng *et al.* 2007; Hopkins *et al.* 2009; Poelman *et al.* 2010; Soler *et al.* 2012). *Brassica*ceous plants contain glucosinolates (GLS) that are a group of well-studied plant secondary metabolites

(Halkier & Gershenzon 2006). Upon tissue damage, GLS are exposed to the myrosinase enzyme (possessing a thioglucoside glucohydrolase activity), resulting in the production of several breakdown compounds that may negatively affect a wide range of generalist herbivores (Hopkins *et al.* 2009). However, specialist herbivores on *brassica*ceous plants are well adapted to GLS-containing plants and have evolved specific detoxification strategies (Wittstock *et al.* 2004). In response to herbivore attack, *brassica*ceous plants also release HIPVs that have been shown to be attractive to natural enemies and to enhance their foraging efficiency (Bruce *et al.* 2005; Dicke & Baldwin 2010; Hare 2011; McCormick *et al.* 2012).

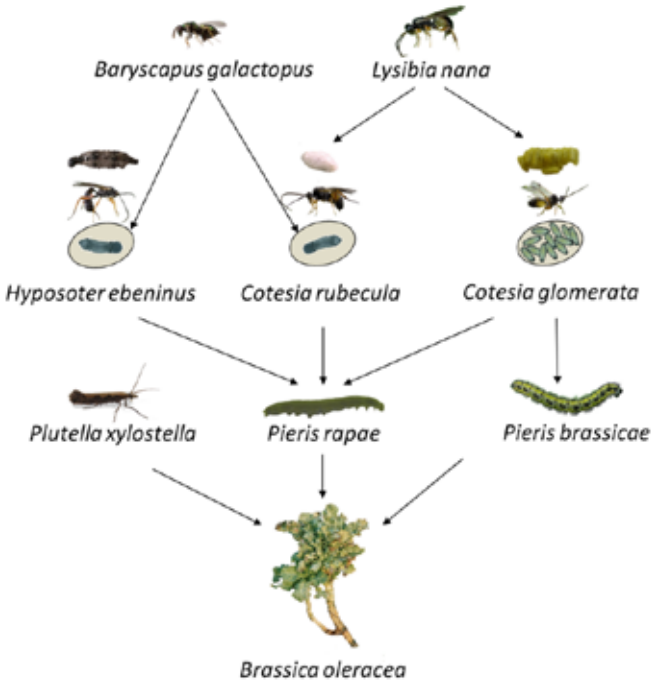


Figure 1. The four-trophic-level system used in this research program.

The Laboratory of Entomology of Wageningen University has a long history in studying interactions between *brassica*ceous plants and insects (Broekgaarden *et al.* 2007; Zheng *et al.* 2007; Gols *et al.* 2008; Poelman *et al.* 2010; Gols *et al.* 2011; Soler *et al.* 2012). In this project, I used a cultivar, *B. oleracea* var *gemmifera* cv. Cyrus (Chapter 3), and the wild *B. oleracea* population "Kimmeridge" (Chapters 4 and 5) to study plant-mediated multitrophic interactions. Seeds of the wild *B. oleracea* population "Kimmeridge" were collected from several plants growing along the south coast of the United Kingdom, near Swanage in Dorset (Gols *et al.* 2008). This population has strong induced responses to specialist herbivore infestation (Gols *et al.* 2008). Moreover, HIPVs emitted by Kimmeridge plants were shown to be attractive to parasitoids (Gols

et al. 2011). The model plant species *Arabidopsis thaliana* (L.) Heynh. (*Brassica*ceae) that is widely used in plant sciences (plant genetics, evolution and development) belongs to the same family (Mitchell-Olds 2001). Due to its close phylogenic relationship, genomic databases for *A. thaliana* can be used as references for transcriptomic studies of *B. oleracea* (Chapter 4).

Insect herbivores

In nature, cabbage white butterflies *Pieris rapae* L. (Lepidoptera: Pieridae) and *P. brassicae* L. (Lepidoptera: Pieridae) are both specialist herbivorous insects that co-occur on *Brassica* plants. Female butterflies of *P. rapae* lay a single egg with each oviposition event and larvae feed solitarily. *P. rapae* is known to be well-adapted to glucosinolate-containing host plants with its highly evolved detoxification strategies to prevent formation of toxic isothiocyanates (Wittstock *et al.* 2004). *Pieris brassicae*, known as the Large Cabbage White butterfly, is a gregarious species. Female butterflies of *P. brassicae* lay clutches of up to 100 eggs. Young larvae feed gregariously until they reach the fourth instar. Both *Pieris* species have been widely used in studies of herbivore-induced responses in *brassica*ceous plants and plant-mediated tritrophic interactions (Mattiacci *et al.* 1995; Brodeur *et al.* 1998; De Vos *et al.* 2005; Broekgaarden *et al.* 2007; Poelman *et al.* 2011b).

In addition, the Diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) was used as an indicator of herbivore-induced plant phenotypic changes (Chapter 5). It is known that female *P. xylostella* moths prefer to oviposit on plants that have been damaged by other herbivores and that it is sensitive to small phenotypic changes in induced plants (Poelman *et al.* 2011b).

Parasitoids

The primary parasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae) is a gregarious koinobiont that lays about 15-60 eggs per host and the host continues to feed until fully grown parasitoid larvae emerge from the host in its fifth instar (Geervliet 1997). Thereafter, *C. glomerata* larvae spin yellowish silk cocoons and pupate. Adult wasps are free-living and feed on sugar sources. *Cotesia glomerata* is considered to be specialist on *Pieris* caterpillars. In the Netherlands, larvae of *C. glomerata* are able to successfully develop and are frequently found in both *P. rapae* and *P. brassicae* caterpillars (Geervliet 1997).

In addition, two solitary parasitoids, *Cotesia rubecula* Marshall (Hymenoptera: Braconidae) and *Hyposoter ebeninus* Gravenhorst (Hymenoptera: Ichneumonidae), were also included in the study system. Both species are able to parasitize *P. rapae* and lay a single egg per host. Parasitism by *C. rubecula* and *H. ebeninus* leads to a developmental arrestment of the host in the third or fourth instar (Harvey *et al.* 1999; Harvey *et al.* 2010; Poelman *et al.* 2011b).

Hyperparasitoids

The highly diverse hyperparasitoid community represents a major share of organisms at the fourth tropic level in the food webs involving *brassica*ceous plants and their associated consumers (Godfray 1994; Sullivan & Volkl 1999; Poelman *et al.* 2012). These parasitic wasps are the enemies of natural enemies of herbivores. Two major groups of hyperparasitoids have been described according to the host developmental stages that hyperparasitoids attack. Firstly, primary hyperparasitoids oviposit in the larvae of primary parasitoids that develop inside their caterpillar host. *Baryscapus galactopus* Ratzeburg (Hymenoptera: Eulophidae) is a gregarious koinobiont that lays up to 30 eggs inside a single parasitoid larva, and its larvae develop inside the parasitoid hosts that continue to feed and grow within their own herbivore host (Harvey *et al.* 2012). *Baryscapus galactopus* widely occurs in Eurasia and is able to hyperparasitize all three parasitoid species mentioned above (Chapters 6 and 7). Unlike *B. galactopus*, *Mesochorus gemellus* Holmgren (Hymenoptera: Ichneumonidae) is a solitary primary hyperparasitoid and parasitizes the two *Cotesia* species, but cannot develop in *H. ebeninus*.

Furthermore, secondary hyperparasitoids are ectophagous and attack pupae of their hosts. In this thesis, *Lysibia nana* Gravenhost (Hymenoptera: Ichneumonidae) is an important seconddary hyperparasitoid of the two *Cotesia* species (Chapters 3-5). *Lysibia nana* oviposits a single egg on the parasitoid pupa in each host cocoon.

Thesis outline

In Chapter 2 the effects of herbivore-associated organisms (HAOs) on plant responses to herbivory are discussed. Recent progresses in the study of how plants respond to integrated stressors are reviewed. In addition, this chapter provides evidence that HAOs are able to directly affect plant responses to herbivory. Furthermore, HAOs may indirectly influence plant responses via altering the herbivore's foraging behaviour and physiological status. At the end, I speculate that HAOs may serve as potential driving force of plant-insect coevolution and propose future perspectives on using genomic tools in the study of plant-insect relationships as interactions among communities rather than individuals.

In chapter 3, I present field experiments on the specificity of hyperparasitoids using HIPVs emitted by cultivated *B. oleracea* for host location under natural conditions. In addition, I address the question whether hyperparasitoids have preferences towards HIPVs induced by unparasitized or parasitized herbivores carrying different parasitoid larvae species. I assessed hyperparasitism ratios in gregarious (*C. glomerata*), or solitary (*C. rubecula*) cocoons that were attached to plants that had received different

herbivore treatments. This chapter also focuses on confirming whether the results from field experiments match the previous laboratory observations of hyperparasitoid preferences for HIPVs of plants damaged by parasitized caterpillars (Poelman *et al.* 2012).

Based on the results described in Chapter 3, I further addressed whether herbivore identity affects foraging preferences of hyperparasitoids. In Chapter 4, a more ecologically relevant wild *B. oleracea* population was selected for studying plant responses to feeding damage by unparasitized or parasitized caterpillars from two different *Pieris* species, using transcriptomics and metabolomics approaches. In addition, I performed Y-tube olfactometer bioassays to test whether the hyperparasitoid *L. nana* responds to HIPVs released by wild *Brassica* plants. I conducted field experiments in two field seasons to assess whether induced plant responses allow hyperparasitoids to locate their parasitoid host in different herbivores.

Chapter 5 addresses how parasitoid larvae developing inside a herbivore indirectly affect plant responses to herbivory and plant volatile-mediated multitrophic interactions. Thus far, several herbivore-associated molecular patterns (HAMPs) that elicit plant responses to herbivory have been identified in herbivore oral secretions (Bonaventure 2012). Therefore, I focused on the labial salivary glands that are a prominent part in caterpillar feeding and their importance for plant defence responses has been demonstrated by using an ablation technique (Musser *et al.* 2006). The differences in profiles of HIPVs induced by intact caterpillars and caterpillars with salivary glands ablated were compared. The effect of these induced plant responses on plant-mediated multitrophic interactions were tested for foraging or oviposition preferences using the hyperparasitoid *L. nana* or the diamondback moth *P. xylostella*, respectively. Finally, transcriptome sequencing approach was used to show the nature of differences in labial salivary glands between unparasitized and parasitized herbivores.

Apart from plant volatiles, there are diverse infochemicals existing in nature, possibly utilized by hyperparasitoids for host location. In Chapter 6, I demonstrate that the primary hyperparasitoid *B. galactopus* can distinguish between body odours of unparasitized and parasitized *P. rapae* caterpillars during the location of their inconspicuous hosts developing in the caterpillar. Furthermore, volatiles from the headspace of unparasitized and parasitized herbivores were collected to identify differences in body odours.

Competitive interactions occur when different species exploit similar niches. This also applies to parasitic wasps (Harvey *et al.* 2013). In nature, frequently different hyperparasitoids are found to use the same parasitoid host (Sullivan & Volkl 1999; Buitenhuis *et al.* 2005; Poelman *et al.* 2012), likely resulting in interspecific competition.

In Chapter 7, a study of intrinsic competition between two primary hyperparasitoids, *B. galactopus* and *M. gemellus* is presented. I specifically addressed which primary hyperparasitoid species is superior in this intrinsic competition, as well as whether the sequence of hyperparasitism affects the outcome of intrinsic competition among hyperparasitoid larvae.

Finally, the findings of this thesis are discussed in Chapter 8, with an emphasis on community-wide consequences of herbivore-induced plant responses using multi-disciplinary approaches. Chapter 8 focuses on how the extended phenotype of parasitoids through their herbivore host in turn affects the plant phenotype, resulting in unexpected species interactions that may challenge the “cry for help” hypothesis in plant indirect defence. The effects of HAOs on plant-mediated multitrophic interactions are emphasized and I conclude that the effect of HAOs on the structure of the plant-associated insect community are important for our understanding of the dynamics of such communities. Ultimately, I provide an outline for future directions in expanding studies of plant-insect interactions from interactions between individuals to interactions between communities.

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Chapter 2

Insect herbivore-associated organisms affect plant responses to herbivory

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Abstract

In nature, plants interact with many organisms and need to integrate their responses to these diverse community members. Knowledge on plant-insect relationships has accumulated rapidly during the last decades. Yet most studies on direct or indirect defences of plants against herbivory have treated herbivores as individual stressors. However, herbivores often consist of communities themselves, comprising organisms such as parasites and symbionts, which may have important effects on the herbivore phenotype, and consequently on interactions of the herbivore with its food plant. Here, we review how herbivore-associated organisms affect plant-herbivore interactions. Organisms associated with herbivores can directly affect how a plant interacts with their herbivorous hosts, by interfering with plant signal-transduction pathways, repressing the expression of plant defence-related genes, or altering plant secondary metabolism. In addition, herbivore-associated organisms can also affect plant responses indirectly by their effect on the behaviour and physiology of their herbivore host. The changes in plant phenotype that arise from herbivore-associated organisms may subsequently affect interactions with other community members, thereby impacting community dynamics. Furthermore, herbivore-associated organisms may act as a hidden driving force of plant-herbivore coevolution. Therefore, to understand plant-herbivore interactions it is important to realize that every single herbivorous insect constitutes a community in itself.

Keywords: extended phenotype, herbivore-associated organism (HAO), herbivory, insect-plant interactions, parasite, parasitoid, plant defence, symbiont.

Introduction

As members of diverse ecological communities, plants and insect herbivores have coevolved for *c.* 350 million yr. Insects are the most speciose group of organisms on the planet, and *c.* 50% of them feed on plants (Schoonhoven *et al.* 2005).

In natural ecosystems, plants interact with many organisms simultaneously, which may influence the pairwise interactions between plants and insects profoundly (Figure 1a; Stout *et al.* 2006; Stam *et al.* 2014). When a plant is attacked by multiple attackers, the responses of the plant to the individual attackers may interact and consequently result in unique plant responses based on the order of colonization, type of feeding behaviour and time lag between arrival of the attackers (Voelckel & Baldwin 2004; Stam *et al.* 2014). In fact, plants are not alone when interacting with herbivores. Organisms associated with plants may affect the interactions between plants and herbivores either positively or negatively (Philippot *et al.* 2013). The presence of a microbial community on plant roots may affect the growth and defence phenotype of a plant and thereby influence multitrophic interactions in the rhizosphere and plant-mediated below-ground-above-ground species interactions (Oldroyd 2013). Some plant-associated organisms, such as endophytes, are even integrated in plants and provide plants with additional defence properties against insect herbivory (Kogel *et al.* 2006). Therefore, plants and their associated organisms constitute a community that is faced with the challenges imposed by herbivores.

It has often been ignored in studies of plant-herbivore interactions that each individual herbivore also represents a community in itself, consisting of different herbivore-associated organisms (HAOs; Figure 1b). Yet it is well known that all higher organisms are complexes of many species that live in symbiosis and which may determine the phenotype of the individual with which they are associated (Gilbert *et al.* 2012). For insect herbivores, for example, it is well known that aphids harbour important endosymbiotic bacteria that provide them with nutrients, protect them against parasitism or aid them in dealing with plant defences (Douglas 2009; Frago *et al.* 2012). The composition of the herbivore-associated community may be af-fected by the secondary compounds of the herbivore's food plant (Kohl & Dearing 2012). However, in addition, HAOs may also influence plant responses to insect herbivory. One of the major groups of HAOs consists of insect parasites that live in or on their host and extract resources from it, leading to a loss of host fitness (Hughes *et al.* 2012). The insect parasites can be micro-organisms (such as fungi and bacteria), viruses or macro-organisms (such as parasitic worms and parasitic wasps; Figure 1b; Hughes *et al.*, 2012). Insect parasites have evolved remarkable strategies to manipulate their host's development, physiology, morphology, evolution and ecology (van Houte *et al.* 2013). Yet other HAOs may be beneficial to herbivores, such as the endosymbiotic microbes of aphids

(Douglas 2009). The presence of HAOs often results in an extended phenotype of the insect host, and this may affect induced responses of plants to feeding damage by their insect host. There is growing evidence of the importance of HAO in plant-insect interactions, which suggests that we should consider the herbivore and its hidden associated community of HAOs as an integrated stressor that interacts with the plant (Figure 1c; Frago *et al.* 2012; Hughes *et al.* 2012).

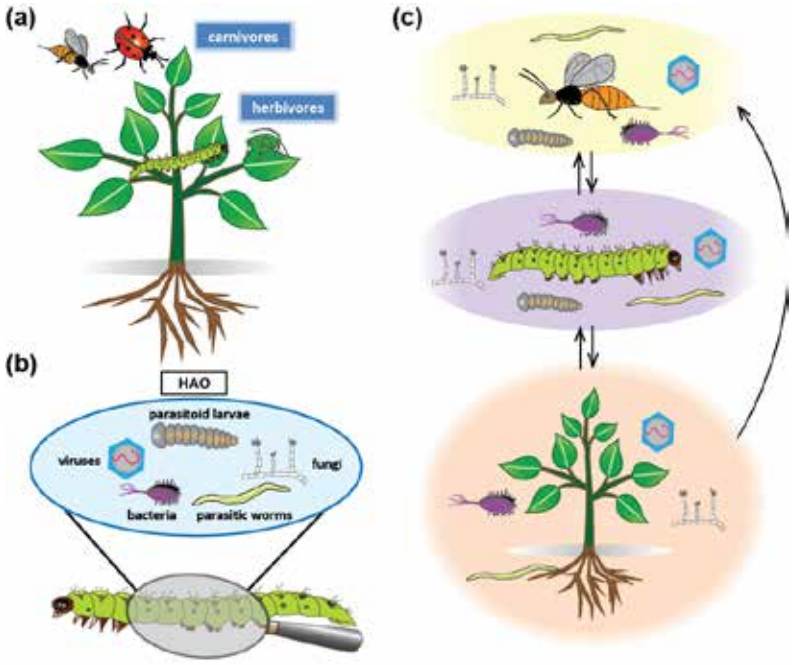

Figure 1. Each member of an ecological community represents a community in itself. (a) a simplified tritrophic community, where the community members are considered as 'individuals'. (b) herbivorous insects consist of communities themselves, comprising both macro- and micro-organisms. (c) interactions in a tritrophic community are not interactions among 'individuals', but in fact interacttions among communities. HAOs: herbivore-associated organisms.

In this review, we discuss recent progress in the study of plant-insect interactions, with a focus on how plants deal with integrated stressors of herbivores and their associated organisms. We review how HAOs directly affect plant responses to herbivory; how HAOs indirectly affect plant responses to herbivory by affecting herbivore behaviour and physiology; how plant responses to integrated stressors result in altered interactions of plants with other community members; and whether HAOs are involved in plant-insect coevolution. Finally, we provide future directions for studying these interactions using genomics tools.

HAOs directly affect plant responses to herbivory

To cope with attack from herbivores, plants have evolved sophisticated direct and indirect defences (Karban & Baldwin 1997; Schoonhoven *et al.* 2005; Heil 2008; Dicke & Baldwin 2010; Kessler & Heil 2011). To activate defence responses, plants recognize insect attack by their damage pattern and by perceiving herbivore-derived chemical cues, such as herbivore-associated elicitors or herbivore-associated molecular patterns (HAMPs; Bonaventure 2012). The elicitors induce signal-transduction pathways regulated by phytohormones and gene transcripts that modulate herbivory-induced responses in plants (Erb *et al.* 2012; Pieterse *et al.* 2012). HAOs may come into contact with plants and affect the induction of plant defence responses, secondary metabolism, physiological status and, consequently, plant-herbivore interactions (Kaiser *et al.* 2010; Chung *et al.* 2013; Luan *et al.* 2013).

Plants are able to induce specific responses to herbivory, affected by the identity of the attacker. These finely tuned induced plant responses can depend on the specialization and feeding guild of the insect herbivores (Voelckel & Baldwin 2004; Ali & Agrawal 2012; Zhang *et al.* 2013). Several HAMPs that plants use in herbivore recognition have been identified in the regurgitant (Bonaventure 2012) of caterpillars that come into contact with plants during herbivore feeding (Vadassery *et al.* 2012). However, plant wounds are open not only to herbivore elicitors, but also to the community of microorganisms that inhabits the foregut of these caterpillars (Figure 2a). Moreover, these microorganisms may be expected to influence HAMP-dependent herbivore recognition, just as parasitic wasps developing in a herbivore may influence elicitor-mediated plant responses (Poelman *et al.* 2011b). In addition, an increasing number of studies indicate that numerous insect species vector plant viruses or pathogenic bacteria and fungi that may influence plant responses to herbivory (Stout *et al.* 2006; Luan *et al.* 2013). Insects may benefit from vectoring plant pathogens, because the induced defence of plants against pathogens often interferes with the induced defence against insects. This is a result of the antagonistic cross-talk between the signal-transduction pathways activated in response to herbivore and pathogen attack (Stout *et al.* 2006; Thaler *et al.* 2012). For instance, *Bemisia tabaci* whiteflies perform better on tobacco plants infected with begomovirus that is vectored by the insects. The enhanced performance of insects is the result of suppression of the biosynthesis of major defence compounds, particularly terpenoids (Luan *et al.* 2013). Interestingly, antibiotic-treated Colorado potato beetle (*Leptinotarsa decemlineata*) larvae lose the ability to suppress antiherbivore defences in tomato; extensive analyses show that microbial symbionts residing in the beetle's oral secretions are involved in defence suppression (Chung *et al.* 2013). When honeydew excreted by aphids drops onto the plant, it may suppress defence-related jasmonic acid accumulation by inducing salicylic acid, suggesting that bacteria within the honeydew may make plants less

resistant to the aphids (Figure 2a; Schwartzberg & Tumlinson 2014). Moreover, a transcriptomics analysis of maize plants revealed that defence-related genes were down-regulated by feeding of beetles carrying endosymbiotic *Wolbachia* bacteria (Barr *et al.* 2010). The insect vectors can also benefit from virus-infected plants with increased growth rates, and consequently a reduced period of vulnerability to predation (Belliure *et al.* 2008). Although the virus may benefit its insect vector by suppressing plant defensive responses, it may cause negative effects on nonvector insects that feed from the same plant (Donaldson & Gratton 2007).

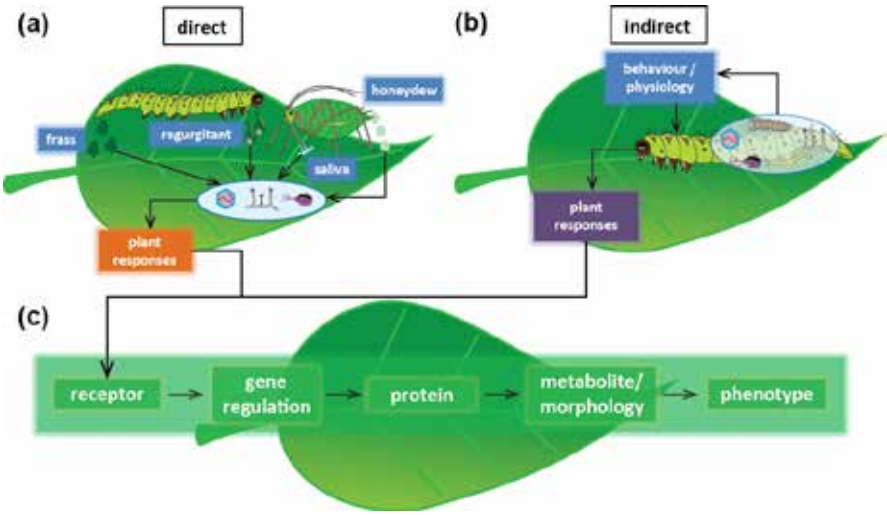

Figure 2. Direct and indirect effects of herbivore-associated organisms (HAOs) on plant responses to herbivores. (a) HAOs carried by herbivore frass, honeydew, regurgitant or saliva can directly affect plant responses to herbivory; (b) HAOs can also indirectly influence plant responses by manipulating behaviour and physiology of their herbivore host; (c) the presence of HAOs influences perception of herbivore attack by plants, thus altering plant phenotype.

Herbivore-associated organisms may also influence plant-insect interactions by altering the emission of plant volatiles that make plants apparent to other community members and consequently play an important role in interactions within ecological communities. This has mainly been studied in tripartite pathogen-insect vector-plant interactions. Increasing evidence indicates that bacterial pathogens and viruses are able to alter the foliar and floral volatile emissions of their host plants, consequently enhancing both vector recruitment to infected plants and subsequent dispersal to healthy plants, thus revealing pathogen-insect mutualisms (Mauck *et al.* 2010; Shapiro *et al.* 2012). Compared with effects of pathogen-induced plant volatiles on vector attraction, little is known about how nonvectors and community members from other trophic levels respond to these induced changes in plant volatiles, but it is likely that other community members respond to these induced changes in the plant phenotype (Dicke & Baldwin 2010).

HAOs indirectly affect plant responses to herbivory

Herbivore-associated organisms may also interact indirectly with host plants via their herbivore host, without physical contact of the HAO with the plants. HAOs are well known for their host manipulation abilities, both behaviourally and physiologically (Figure 2b; Godfray 1994; Hughes *et al.* 2012; van Houte *et al.* 2013), resulting in extended phenotypes of their herbivore hosts. Thereby, the presence of HAOs could lead to altered herbivore traits that might affect plant responses to herbivory (Figure 2b).

HAOs influence host behaviour

The presence of HAOs often leads to changes in the behaviour of the host insect, including reproduction, feeding behaviour and locomotion (Hughes *et al.* 2012). Parasite-induced changes in host behaviour are often thought to increase the fitness of the parasite and may be actively driven by the parasite (Lefevre *et al.* 2009). Changes in movement of hosts as a result of parasitism have been well investigated in different parasite–host systems (van Houte *et al.* 2013). For instance, fungal or viral infections may manipulate the behaviour of their insect host such that the host now moves to the top of the canopy, which is beneficial for reproduction and spread of the parasites (Hoover *et al.* 2011). Also, parasitic worms or wasps manipulate host movement for their own benefit (Godfray 1994; Biron *et al.* 2006; Libersat *et al.* 2009; van Houte *et al.* 2013). Because leaves at different positions in a plant may differ in their responses to herbivory (Rostas & Eggert 2008), differential distribution of feeding by infected vs uninfected herbivores may result in differential spatial arrangements of the induced plant phenotype, which may consequently affect other attackers.

In some extreme cases, parasitoids manipulate their host to the extent that it becomes a ‘bodyguard’ that physically protects the parasitoids against subsequently approaching predators (Harvey *et al.* 2008). Parasites can also induce changes in the feeding behaviour of their host, and such effects are often specific to the species of parasite developing in the herbivore (Godfray 1994; Poelman *et al.* 2011a). Because feeding behaviour characteristics influence plant responses (Mithofer *et al.* 2005), parasites may indirectly influence plant responses through their effects on feeding behaviour.

Little is known about the molecular mechanisms behind manipulations of host behaviour by parasites; the available knowledge has been gained primarily from model systems using viral parasites (van Houte *et al.* 2013). For more complex organisms, genes and/or proteins of other parasites (such as bacteria, fungi, parasitic wasps) involved in behavioural manipulation of the host have been less well studied. However, the observed changes in herbivore movement patterns show high similarity

across different groups of HAOs. These similarities indicate that mechanisms behind host manipulation may be highly conserved among parasites to maintain their parasitic life history (Ponton *et al.* 2006). On the other hand, similar patterns in behavioural changes in host herbivores may also indicate conserved strategies of the hosts in response to parasitism. Future studies are required to unravel why and how the parasites alter their host's behaviours.

HAOs influence host physiology

In addition to host behaviour manipulation, HAOs also alter host physiology. The presence of HAOs can affect host development. When developing in their herbivore host, parasitoids influence host growth by interfering with the production of juvenile hormone and ecdysone of their host, which are responsible for maintaining the juvenile characters of the host and initiate moulting to the next larval instar, respectively (Godfray 1994). Parasitoids may induce their hosts to stay longer in the larval feeding stage, which has been shown for the gregarious parasitoid *Cotesia congregata*, which parasitizes tobacco hornworm (*Manduca sexta*) caterpillars (Godfray 1994). The parasitoid prevents metamorphosis of its host larva by suppressing the drop in juvenile hormone production before pupation, leading to a sixth supernumerary larval stage. This prolonged feeding stage of the host is beneficial to the parasitoid larvae, allowing them to acquire more nutrients. By contrast, the solitary parasitoid *Cotesia rubecula* arrests the growth of its host, caterpillars of *Pieris rapae*, in the third or fourth larval instar (Harvey *et al.* 1999). These changes in host physiology affect feeding rate and might thus affect plant responses to herbivory. Parasitoid species could even have a further unique effect on their herbivore host's physiology, by altering the herbivore's oral secretion, which plays a vital role in eliciting plant responses (Poelman *et al.* 2011b).

Symbiotic microbes of insect herbivores could also contribute significantly to modulation of host physiology. Microbial symbionts can provide essential nutrients to the host, such as amino acids, vitamins and sterols (Douglas 2009). Symbionts of herbivorous insects could greatly improve nutrient uptake and open niches to their insect host, allowing colonization of a broad range of host plants (Douglas 2009). The identity of microbial symbionts of phloem-feeding herbivores affects the capacity of the herbivores to switch between food plant species (Tsuchida *et al.* 2004; Oliver *et al.* 2010). Microbial symbionts may also contribute to herbivore resistance to insecticides; for example, susceptibility to insecticides in the silverleaf whitefly *Bemisia tabaci* depends on the density of endosymbionts (Ghanim & Kontsedalov 2009). Whether these effects of microbial symbionts on herbivore physiology affect plant responses to herbivory remains to be investigated.

Similar to physiological modulations, the immune system of an insect herbivore is not only regulated by the herbivore itself but also by HAOs. Insects largely depend on their immune system to combat invasions by other organisms. HAOs could provide their host with protection against a wide range of natural enemies (Oliver *et al.* 2014). Some symbionts can directly protect the host from attack by natural enemies by producing toxins or deterrents (Hansen *et al.* 2012). Some others provide host protection indirectly by modulating the host immune system, such as *Wolbachia* bacteria that promote host resistance to viral infection in *Drosophila* fruit flies, resulting in protection of the fruit flies against a wide range of RNA viruses (Hedges *et al.* 2008). Although the mechanisms underlying host immune system modulation by HAOs remain to be further investigated, these direct and indirect protections provided by HAOs are likely to contribute to the ability of insect herbivores to overcome the challenges imposed by their food plants and their natural enemies (Oliver *et al.* 2010; Frago *et al.* 2012).

The extended phenotype of the herbivore that results from the HAO-induced behavioural and physiological manipulations affects the interaction of the herbivore with its food plant (Figure 2b). Manipulations by HAOs of host-feeding behaviour, including amount of food consumed, feeding pattern and shifts in feeding sites (between old and young tissues or vegetative and reproductive tissues), could have important consequences for plant growth and defence responses. Moreover, the physiological changes that are expressed in the host's oral secretions affect recognition of the attacker and induced plant responses (Poelman *et al.* 2011b). The behavioural and physiological manipulations by HAOs could further indirectly affect plant responses to herbivory.

Community-wide consequences of HAO-mediated changes in plant–herbivore interactions

The fact that HAOs can manipulate the herbivore's phenotype, and consequently the herbivore's interaction with its food plant and the plant's responses, means that HAOs affect the plant phenotype (Figure 2c). That these HAO-induced plant phenotypes result in altered inter-action networks of the herbivore hosting the HAOs and other plant-associated organisms has been well established for interactions between parasitoids and their herbivore host. The larvae of parasitic wasps that feed within their herbivorous host do not have physical contact with host plants. Yet, in a parasitoid species-specific manner, they affect the growth of the herbivore as well as the composition of its oral secretion and thereby interact with the host plant through their herbivorous hosts. The HAO-mediated altered composition of the oral secretions induced defence-related genes and volatile emissions differentially for the

presence or absence of parasitoid larvae. Moreover, the parasitoid species can have a more pronounced effect on plant gene transcription than the herbivore species in which the parasitoid resided (Poelman *et al.* 2011b). The changes in plant phenotype subsequently affected foraging behaviour and performance of insects at the second up to the fourth trophic level (Poelman *et al.* 2011a; Poelman *et al.* 2011b; Poelman *et al.* 2012). For instance, the herbivore *Plutella xylostella* (the diamondback moth) exhibits an altered oviposition preference: the moths preferred to oviposit on plants infested with unparasitized caterpillars than on plants infested with caterpillars parasitized by a parasitic wasp that cannot attack *P. xylostella* (Poelman *et al.* 2011b). In addition, plant responses induced by parasitoid larvae that develop within their host herbivore can also be perceived by top consumers at the fourth trophic level (Poelman *et al.* 2012). The hyperparasitoid wasp *Lysibia nana* differentiates between the blends of plant volatiles induced by unparasitized herbivores and herbivores carrying parasitoid wasp larvae, and uses this to successfully locate their hosts. These interaction networks that are driven by direct and indirect effects of HAOs on plant traits are likely to be found for other tritpartite systems, such as virus-herbivore-plant or symbiont-herbivore-plant associations as well. Because plants are the basis of food chains in terrestrial ecosystems, phenotypic changes in plants may significantly influence the community structure and dynamics through bottom-up effects (Bukovinszky *et al.* 2008), and thus HAO effects on plant phenotypes have a strong potential to shape community processes.

HAOs as a potential driving force of plant-insect coevolution

Although it was previously known that some HAOs are able to manipulate host behaviour and physiology, we are now beginning to realize that these HAOs also play a role in the interactions between the their host and the food plants of their host. Unexpected interactions are being recorded between herbivores and their host plants when HAOs are considered as components of the plant-herbivore interaction (Poelman *et al.* 2011b; Frago *et al.* 2012; Chung *et al.* 2013; Luan *et al.* 2013). The emerging view is that plant-insect interactions across different trophic levels in food webs are more complex than commonly considered. The presence of HAOs may interfere with the plant to recognize its herbivore, for example, through interference with signal transduction in the plant and with defence responses. Thus, HAO-mediated effects result in extended phenotypes in plants. It is likely that HAOs are even involved in the coevolutionary arms race between herbivores and plants. Therefore, a major question concerns the driving force in herbivore-plant coevolution: is it the herbivore itself, the HAO, or the combination of herbivore plus its HAO as an integrated stressor? This is likely to have important consequences for our view

on the evolution of plant-herbivore interactions. For instance, instead of evolving an adaptation to a plant defence through, say, enzymatic breakdown of a plant toxin, a herbivore could also evolve to interact with a new HAO that eliminates the effects of the plant defence. For instance, the lack of endosymbionts in the weevil *Sitophilus linearis* can be associated with a switch from feeding on nutrient-poor host plants to feeding on plants that provide a higher nutritional value (Clark *et al.* 2010). Moreover, genetic changes in the endosymbiont may affect selective pressures on the insect host (Clark *et al.* 2010). The herbivore may benefit from microbial evolution that results in microbial genotypes that evolve to deal with plant secondary metabolites. After all, a selective advantage for the herbivore also favours its endosymbionts. Because generation times of microbes are much shorter than generation times of insects, this may mean that adaptation can be even faster.

Future perspectives

Most of the studies addressing herbivore-induced plant responses have been based on the assumption that herbivores interact with their host plants as 'individuals' (Gilbert *et al.* 2012). However, the phenotype of the herbivore that interacts with the plant is a complex community in itself and each of the community members can influence the herbivore's phenotype (Figure 1b). The emerging view that HAOs have important effects on herbivore behaviour, development and a herbivore's interactions with host plants, gives rise to new research directions in the field of the evolutionary biology of plant-insect interactions.

The knowledge emerging from studies of tripartite microbe-plant-insect interactions, insect-microbe symbiosis and herbivore-induced plant volatiles (HIPV)-mediated plant-hyperparasitoid interactions urges us to consider HAOs as important hidden players in plant-insect interactions and to study the effects of HAOs on plant responses to herbivory (Frago *et al.* 2012). So far, studies have focused, in particular, on the effects of individual HAOs on host manipulations and plant-herbivore interactions. Because the herbivore constitutes a community of HAOs in itself, one of the challenges is to assess HAO composition and identity and their effects on the phenotype of their insect hosts. Metagenomic approaches provide excellent opportunities to characterize the entire microbiota that reside in or on herbivorous insects (Philippot *et al.* 2013). Further analysis of these communities will yield insight into HAO diversity and dynamics, as well as the interactions among HAOs, which could profoundly influence not only the phenotype of the herbivorous host but also that of the food plant of the herbivore.

To understand the role of HAOs in host manipulation, recent advances in genomics and proteomics provide reliable tools to study host-parasite interactions from the

level of the individual to unravelling the underlying molecular mechanisms (Biron *et al.* 2006; Lefevre *et al.* 2007). Through this approach, host manipulation by specific parasites can be studied, and mechanisms can be compared between insect-HAO combinations. Apart from direct effects of HAOs on the herbivore, direct and indirect effects of HAOs on plant responses to herbivory can also be addressed. By comparing plant metabolome and transcriptome profiles in response to herbivores with or without HAOs, for example, the effects of HAOs on herbivore-induced plant responses can be investigated. With rapidly advancing sequencing techniques, we are no longer restricted to model species; genomic information for many other nonmodel but ecologically relevant organisms will become available and will aid studies in this field.

Although it is now recognized that plants are able to respond specifically to different attackers, we will never fully understand how plants cope with herbivores as integrated stressors when the effects of HAO are ignored. Each member of the plant-insect community constitutes a community in itself; therefore, studies of plant-insect interactions in fact address the interactions among different communities rather than interactions between individual organisms (Figure 1c). Although there is a lack of information on associated organisms in the community members at the third or higher trophic level (Dicke 1996), at least some viruses associated with parasitic wasps are known to be involved in parasitoid-host interactions (Harvey *et al.* 2013), and ant-associated bacteria are known to contribute to ant-plant defensive mutuallisms (Gonzalez-Teuber *et al.* 2014). The changes in plant phenotype that are induced by a herbivore holobiome (*sensu* Gilbert *et al.* 2012) will affect other community members at different trophic levels and exert 'bottom-up' effects on the structure of the plant-insect metacommunity.

Combining information from different disciplines and at different degrees of biological complexity will deepen our understanding of how HAOs affect plant phenotypes through the manipulation of their insect host, resulting in community-wide consequences for HAO-plant interactions. It is not only herbivores that constitute communities; in fact, every macro-organism constitutes a community that includes microorganisms (Gilbert *et al.* 2012). This means that the units within food webs and communities of macro-organisms are actually all communities rather than individuals. Expanding studies of plant-insect interactions from interactions between individuals to interactions between communities raises fundamental questions on the key species that drive the system. This makes the study of plant-insect inter-actions more complex but definitely also more intriguing. Realizing that organisms often do not act as individuals will be the start of new, exciting developments in this research field.

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Chapter 3

Hyperparasitoids use herbivore-induced plant volatiles in host location under field conditions

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Abstract

Herbivore-induced plant volatile (HIPVs) mediated plant-insect interactions have been extensively studied within systems consisting of three trophic levels. However, plant-insect food webs generally include four or more trophic levels. Hyperparasitoids at the fourth trophic level are parasitic wasps that attack larvae and pupae of primary parasitoids. Thus far, little is known about host-location behaviour of hyperparasitoids. Here, using a field experiment, we demonstrate that hyperparasitoid wasps take advantage of the odours that plants produce in response to feeding by caterpillars to locate their host. Under field conditions, we found higher hyperparasitism rates on plants that were infested with caterpillars parasitized by the gregarious parasitoid *Cotesia glomerata* compared to infestation with healthy unparasitized caterpillars. Our results show that hyperparasitoids can reliably use HIPVs induced by parasitized caterpillars to locate their host. We concluded that the effects of herbivore-induced plant volatiles should be placed in a community-wide perspective that includes species at the fourth trophic level to further improve our understanding of the ecological consequences of volatile release by plants. Furthermore, these findings suggest that the impact of species at the fourth trophic level should also be considered when developing Integrated Pest Management strategies aimed at optimizing the control of insect pests using parasitoids.

Keywords: *Brassica oleracea*, herbivore-induced plant volatiles, hyperparasitoid, parasitoid, *Pieris rapae*, multitrophic interaction.

Introduction

Plant volatiles play an important role in interactions within ecological communities. The emission of plant volatiles makes plants apparent to other community members, including herbivorous insects and their natural enemies, as well as neighbouring plants (Vet & Dicke 1992; Baldwin *et al.* 2006; Dicke & Baldwin 2010; Pierik *et al.* 2014). Plant-volatile mediated interactions have been well studied within the context of three trophic levels (Dicke & Baldwin 2010; Vet & Dicke 1992). Plants release so-called herbivore-induced plant volatiles (HIPVs) in response to attack by herbivorous insects. HIPVs have been found to attract natural enemies of herbivores at the third trophic level, such as primary parasitoids (Dicke & Baldwin 2010; Godfray 1994; Schoonhoven *et al.* 2005; Vet & Dicke 1992). Therefore, releasing HIPVs has been considered a plant indirect defence and hypothesized to benefit plant fitness (van Loon *et al.* 2000; Hoballah & Turlings 2001; Kessler & Heil 2011; Schuman *et al.* 2012b). Besides attracting natural enemies of herbivores, induced plant volatiles may also influence interactions between plants and other community members, which may consequently affect the fitness benefits of volatile emission (Kaplan 2012). Natural food webs generally consist of four or more trophic levels. The enemies of parasitoids, hyperparasitoids at the fourth trophic level, have not been included in the debate on the plant fitness benefit of volatile release because little is known about their host-location behaviour.

Hyperparasitoids are parasitic wasps attacking larvae or pupae of primary parasitoids (Sullivan 1987). They comprise a major share of the fourth trophic level in the insect community. Thus far, little is known about the cues that hyperparasitoids use to find their primary parasitoid hosts (Sullivan & Volkl 1999). The hyperparasitoids are likely to be constrained in locating suitable hosts, as neither the larvae nor the pupae of their primary parasitoid hosts directly feed on the plants (Sullivan 1987). Therefore, primary parasitoids may not directly induce plant responses that give away their presence to hyperparasitoids. For the secondary hyperparasitoids that parasitize the fully cocooned pupae of primary parasitoids, the time window for successful hyperparasitism of pupae is often narrow and restricted to the first few days after the pupae are formed (Harvey *et al.* 2009b). Although parasitoid larvae themselves do not interact with plants, plants have been shown to respond differently to feeding damage by unparasitized or parasitized herbivores (Fatouros *et al.* 2005; Harvey *et al.* 2009b; Poelman *et al.* 2011a; Poelman *et al.* 2011b). Consequently, altered plant responses induced by parasitized herbivores may be further reflected in the plant volatiles emitted. These volatiles may provide hyperparasitoids with reliable information on the presence of their hosts and allow hyperparasitoids to arrive at the cocoons when those have just been formed and are suitable for parasitism (Sullivan & Volkl 1999).

Some species of hyperparasitoids are able to use a range of primary parasitoid species as host, including both solitary (laying a single egg in an herbivore host) and gregarious (ovipositing multiple eggs in an herbivore host) species (McDonald & Kok 1991). It has been suggested that parasitoid larvae may largely affect the physiological status and feeding behaviour of their herbivore host (Godfray 1994; Libersat *et al.* 2009). Moreover, solitary and gregarious parasitoid species may influence their host physiology and feeding behaviour differently, which in turn may induce different plant responses to herbivory (Poelman *et al.* 2011a; Poelman *et al.* 2011b). Hyperparasitoids may prefer one parasitoid host over another, because of the fitness gain in terms of high numbers or quality of offspring when parasitizing specific hosts. Therefore, variation in HIPVs induced by different parasitized herbivores may allow hyperparasitoids to distinguish whether the plant is colonized by herbivores carrying their preferred hosts.

In this study, we used a *Brassica oleracea* based food-web system including four trophic levels, to investigate whether hyperparasitoids are able to locate their primary parasitoid hosts using plant volatiles under field conditions. In the Netherlands, the solitary parasitoid *Cotesia rubecula* and the gregarious *C. glomerata* attack caterpillars of *Pieris rapae* (Small Cabbage White butterfly) that feed on *brassica*ceous plants (Brodeur *et al.* 1998). Fully developed parasitoid larvae emerge from their host and spin a silk cocoon in which they pupate. Individual *C. glomerata* cocoons are generally 40% smaller (in terms of mass) than individual *C. rubecula* cocoons. In terms of the *per capita* fitness potential of the offspring, hyperparasitoids may benefit when developing in pupae of the larger *C. rubecula*. However, the hyperparasitoids may benefit more from finding a caterpillar parasitized by the gregarious *C. glomerata* when considering the cumulative maternal fitness. Therefore, the hyperparasitoids may evolve to respond to cues associated with hosts that provide larger maternal fitness benefits. In previous experiments, we have found that the hyperparasitoid *Lysibia nana* responds to HIPVs under laboratory conditions in a Y-tube olfactometer (Poelman *et al.* 2012). Moreover, *L. nana* responded differently to HIPVs induced by unparasitized or parasitized herbivores, or by herbivores carrying different parasitoid species.

To further test the specificity of hyperparasitoids using HIPVs as cues for host searching in natural conditions, here, using a field study, we specifically addressed the questions: 1) whether hyperparasitoids use HIPVs as cue to locate their primary parasitoid host under field conditions; 2) whether hyperparasitoids have preferences towards HIPVs induced by herbivores carrying different parasitoid larvae.

Materials and methods

Plants and insects

Brassica oleracea var *gemmifera* cv. Cyrus plants used for field studies were grown in 1.45 L pots containing peat soil (Lentse potgrond, no. 4, Lent The Netherlands). They were provided with SON-T light (500 µmol/m2/s; L16:D8) in addition to natural daylight in a glasshouse compartment (18-26 °C, 50-70% RH) for four weeks after germination.

Cultures of *Pieris rapae* and two *Cotesia* species were originally collected from agricultural fields close to Wageningen University, The Netherlands. The hosts were maintained on Brussels sprout plants, *B. oleracea* var *gemmifera* cv. Cyrus, at 23 ± 2 °C under 50-70% RH with a 16:8-h L:D regime. *Cotesia glomerata* were reared exclusively on first-instar (L1) *Pieris brassicae*, whereas *C. rubecula* were maintained on L1 *P. rapae*. To obtain parasitized hosts, several leaves containing host larvae were placed into rearing cages for several hours, then removed and reared in separate cages containing potted Brussels sprouts plants until egression of the parasitoid larvae from their host and pupation outside the host body. Half of the newly formed (within 24h) *Cotesia* cocoons were returned to the main culture and the other half was collected in Petri dishes, and stored at 4 °C to be used in field experiments.

Parasitism protocol

To prepare parasitized caterpillars for the induction treatments on plants in the field, individual L1 *P. rapae* larvae were exposed to a single female *C. glomerata* or *C. rubecula*, which was allowed to parasitize the caterpillar in a glass vial. For *C. glomerata*, caterpillars were considered to be parasitized when wasps had inserted their ovipositor in the caterpillar for at least 5 seconds. For *C. rubecula,* because of herbivore immune responses to parasitoid eggs (Brodeur & Vet 1994), the wasp was allowed to oviposit 3 times in the same caterpillar, to increase the success rate of parasitism. Due to larval interference only a single *C. rubecula* larva would develop eventually (Geervliet *et al.* 2000).

Experimental procedure

Eighty four-week-old plants were transplanted into the field with 1×1m spacing between plants, and allowed to take one week to adjust to field conditions. To induce the plants with different types of herbivory, 20 plants were infested individually with either two unparasitized L1 *P. rapae* caterpillars, or L1 larvae parasitized by either *C. glomerata* or *C. rubecula*. We kept 20 plants undamaged. Unparasitized and parasitized caterpillars were allowed to feed on plants for ten days, which was

approximately the whole development period of *Cotesia* larvae. Each plant was covered with a fine-mesh net when planted to avoid other herbivore infestations from above-ground and to prevent the herbivores used for induction to wander off the plant.

To test the effects of plant induction with different types of herbivory on hyperparasitism, we attached cocoon clutches onto the plants in the field. Individual cocoons of *C. rubecula,* or cocoon clutches of *C. glomerata*, were first attached to a paper disc (3×3 cm) with a small droplet of glue. We removed nets and caterpillars just before attaching the paper discs carrying the cocoons with a pin needle. Half of the plants for each treatment received five *C. glomerata* cocoon clutches, the other half received five *C. rubecula* cocoons. The cocoons were exposed to the natural community of hyperparasitoids and recollected after five days. They were kept separately in 2 ml Eppendorf tubes that were closed with cotton wool. The Eppendorf tubes were checked daily for emerging primary parasitoids and hyperparasitoids. All wasps were identified to species level.

A completely randomized design was applied to the field studies. Five replications were carried out from June until October 2011.

Data analysis

Hyperparasitoid preferences for plant volatiles induced by unparasitized *P. rapae* caterpillars and caterpillars parasitized by gregarious or solitary primary parasitoids under field conditions were analyzed using two Generalized Linear Models (GLM). To analyze the effects of plant inductions with different types of herbivory on hyperparasitism at plant level, we modelled the dependent variable as a binomial occurrence of hyperparasitism per plant, and scored presence of hyperparasitoids in cocoons as 1 and absence as 0. Additionally, to test the effects of the plant inductions on hyperparasitism at cocoon level, we modelled the dependent variable as the number of cocoons or cocoon clutches giving any hyperparasitoid out of the fixed totals of 5 cocoons attached to the plant. Into the two models we included the fixed factors caterpillar induction (undamaged, unparasitized *P. rapae, P. rapae* parasitized by *C. glomerata*, and *P. rapae* parasitized by *C. rubecula*), replicate (five replications), types of cocoons (gregarious or solitary) and the interactions between the three terms.

Results

The re-collected parasitoid cocoons yielded 1083 hyperparasitoids of three species, where 95.5 percent of the total hyperparasitism was contributed by *Lysibia nana. Bathytrix aerea* and *Gelis agilis* were uncommon with 3.4 percent and 1.1 percent respectively (Table 1).

Table 1. Hyperparasitoid species, number and fraction of hyperparasitoid wasps emerging from *Cotesia glomerata* and *C. rubecula* cocoons recollected from field studies.

Hyperparasitoid species	***Lysiba nana***	***Bathythrix aerea***	***Gelis agilis***	**Total**
Number of hyperparasitoids collected	1034	37	12	1083
Percentage of hyperparasitoids collected	95.5	3.4	1.1	100

We found different hyperparasitism rates of cocoons of primary parasitoids on plants damaged by different types of herbivory under field conditions (Table 2). Plants damaged by *C. glomerata* parasitized caterpillars received higher hyperparasitism rates of *C. glomerata* cocoon clutches (Figure 1; Figure 2; Table 2). Infestation of the plant with caterpillars parasitized by *C. glomerata* resulted in nearly 20 percent of attached gregarious cocoons being hyperparasitized, whereas only less than 5 percent of the cocoon clutches attached to undamaged plants were attacked by hyperparasitoids. The cocoons attached to the plants damaged by either *P. rapae* or *C. rubecula*-parasitized *P. rapae* had similar rates of hyperparasitism, around 12 percent (Figure 1). In contrast, solitary cocoons of *C. rubecula* were not differentially hyperparasitized when attached to plants from the 4 different treatments. Volatiles derived from plants damaged by *C. rubecula* parasitized caterpillars led to around 15 percent of hyperparasitism in solitary cocoons (Figure 1).

Table 2. Generalized Linear Model deviance table for the percentage of hyperparasitized cocoon clutches.

	Deviance	**Degrees of freedom**	**P- value**
Full model			
	797.45	399	
Factor			
Caterpillar induction (1)	16.00	3	**0.001**
Replicate (2)	258.83	4	**<0.001**
Type of cocoons (3)	5.53	1	**0.019**
Interaction			
1 * 2	33.48	12	**<0.001**
1 * 3	16.94	3	**<0.001**
2 * 3	10.71	4	**0.030**
1 * 2 * 3	11.17	12	0.514

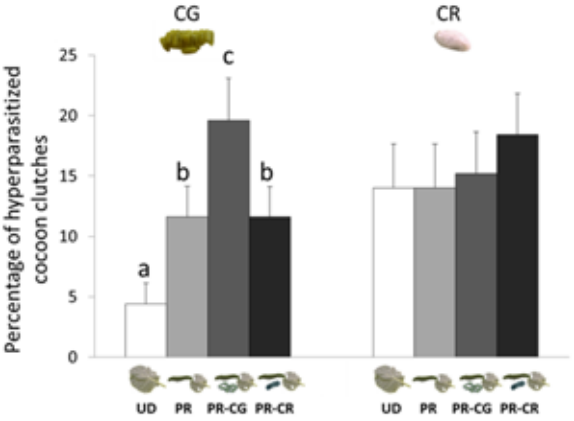

Figure 1. Percentage of *Cotesia* cocoons that resulted in hyperparasitism in the field. The plants were induced with different types of herbivory, including by *Pieris rapae* (PR), *P. rapae* parasitized by *C. glomerata* (PR-CG), *P. rapae* parasitized by *C. rubecula* (PR-CR), or undamaged plants (UD). The left and right groups of bars represent the plants on which we offered cocoon clutches of *C. glomerata* (CG) or cocoons of *C. rubecula* (CR), respectively. Letters indicate significant differences between treatment groups (GLM, $P < 0.05$).

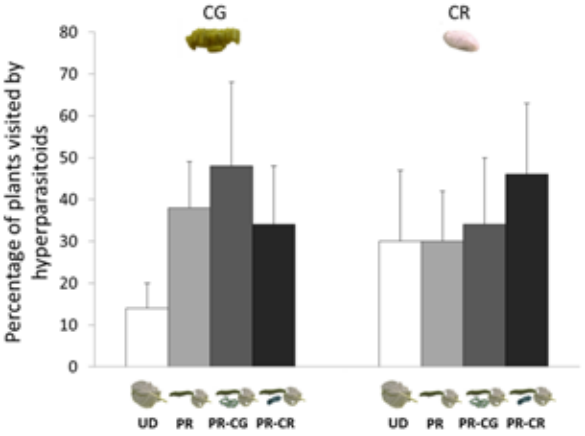

Figure 2. Percentage of plants that were visited by hyperparasitoids in the field. The plants were induced with different types of herbivory, including herbivory by *Pieris rapae* (PR), *P. rapae* parasitized by *C. glomerata* (PR-CG), *P. rapae* parasitized by *C. rubecula* (PR-CR), or undamaged plants (UD). The left and right groups of bars represent the plants on which we offered cocoon clutches of *C. glomerata* (CG) or cocoons of *C. rubecula* (CR), respectively.

Table 3. Generalized Linear Model deviance table for the percentage of plants visited by hyperparasitoids in the field.

	Deviance	Degrees of freedom	P- value
Full model			
	516.71	399	
Factor			
Caterpillar induction (1)	8.32	3	**0.040**
Replicate (2)	157.25	4	**<0.001**
Type of cocoons (3)	0.28	1	0.600
Interaction			
1 * 2	16.92	12	0.153
1 * 3	13.49	3	**0.004**
2 * 3	7.20	4	0.126
1 * 2 * 3	3.93	12	0.985

Boldface type presents significant effects (α=0.05) in a GLM model with a binomial distribution

We also observed that approximately 50 percent of the plants damaged by gregariously parasitized caterpillars gained hyperparasitizations when cocoons of gregarious parasitoids were attached. By contrast, less than 15 percent of the undamaged plants had hyperparasitized cocoons (Figure 2; Table 2). When solitary cocoons were attached, plants received similar percentages of hyperparasitization among different herbivore treatments.

Discussion

Hyperparasitoids are important organisms in terrestrial ecosystems and may significantly shape the structure of the arthropod community (Rosenheim 1998). However, their contribution to multitrophic interactions has often been ignored. In the current study, we specifically investigated whether plant volatiles are used by hyperparasitoids as foraging cues. Previously, it was found that hyperparasitoids responded to HIPVs and preferred plant volatiles induced by *C. glomerata* parasitized caterpillars over healthy caterpillar damage (Poelman *et al.* 2012). Moreover, the volatiles of plants induced by unparasitized and parasitized caterpillars differed in composition. To extend our knowledge on specificity of hyperparasitoids using HIPVs as reliable foraging cues in natural conditions, we carried out field experiments in 2011. This field study demonstrates higher hyperparasitism rates on plants that were damaged by herbivores. Moreover, plants damaged by gregariously parasitized caterpillars showed higher hyperparasitism rates than plants damaged by healthy and solitarily parasitized caterpillars (Figures 1 & 2), matching the previous laboratory findings of hyperparasitoid preferences for HIPVs of plants damaged by parasitized caterpillars (Poelman *et al.* 2012). This match suggests that also in the field the variation in HIPVs induced by healthy and parasitized caterpillars may be reliable cues for hyperparasitoids to locate their host.

However, the effects of our herbivory treatments did not prevail on experimentally applied solitary cocoons of *C. rubecula*. The reasons might be that we offered much larger numbers of individual *C. glomerata* cocoons than *C. rubecula* cocoons, when considering that each *C. glomerata* brood consists of several individual cocoons. Thus, hyperparasitoids may have had a higher chance to explore a large number of individual solitary cocoons, but a limited portion of gregarious cocoon clutches. This may explain why these two types of cocoons shared similar hyperparasitism rates on the brood level, although more hyperparasitoids were recovered from the multiple cocoons of gregarious broods. In addition, due to the setup of this field study, we excluded hyperparasitism by primary hyperparasitoids that parasitize the larvae of the parasitoid hosts. A previous survey of hyperparasitoid presence in *Cotesia* cocoons identified a significant number of primary hyperparasitoids that parasitize *Cotesia*

larvae (Poelman *et al.* 2012). Therefore, the secondary hyperparasitoids might encounter less competition from primary hyperparasitoids, and may therefore more frequently use solitary cocoons as host. Moreover, female hyperparasitoids, such as *L. nana*, carry a limited number of about 40 eggs, and exploit a large proportion of the cocoons once they are able to locate a cocoon clutch (Harvey *et al.* 2011a). The hyperparasitoids locating a gregarious brood may spend longer time on the brood and are egg limited in exploiting the whole brood when the brood size exceed 40, whereas it may cost less time to exploit solitary cocoons (Harvey *et al.* 2011). This may explain why hyperparasitism rates were higher on solitary *C. rubecula* cocoons, but higher numbers of hyperparasitoids were found on gregarious cocoons.

The hyperparasitism rates varied over the field season, which indicates population dynamics of hyperparasitoids over time. Hyperparasitism was generally low in spring and increased towards the end of the season. We also observed a drop and re-rise in hyperparastism ratio from the experiment in August and September, respectively, indicating that many hyperparasitoids may have two or more generations in natural ecosystems. Moreover, we also found a higher hyperparasitism rate of *L. nana* in the solitary cocoons in the field season of 2011 compared to cocoons collected in field seasons of 2005 to 2007 (Poelman *et al.* 2012), indicating year-to-year variation in hyperparasitism ratio.

Herbivorous insects commonly carry numerous other organisms in or on their body, including both micro-organisms and macro-organisms. All of these herbivore-associated organisms potentially affect the behavioural and physiological phenotypes of herbivorous insects. As a consequence, they may alter plant responses to herbivory (e.g. defence-related gene expression and HIPV emission). Recent evidence showed that although primary parasitoid larvae do not directly interact with the food plant, their feeding inside the caterpillar may cause physiological changes in the herbivore host (i.e. composition of regurgitant), and then further indirectly affect plant phenotypes (Poelman *et al.* 2011b; Zhu *et al.* 2014a). To date, several herbivore-associated elicitors that are herbivore-derived chemical cues perceived by plants for activation of a range of defence responses have been identified in herbivore oral secretions (Bonaventure *et al.* 2011). The altered composition of herbivore oral secretion may affect emission of HIPVs, thereby allowing hyperparasitoids to locate their hosts.

It has been well acknowledged that HIPVs enhance foraging efficiency of natural enemies and thereby may benefit the plant as an indirect-defence strategy to defend themselves against insect herbivores. However, it has been intensively discussed whether plant fitness eventually benefits from attracting beneficial insects as bodyguard against herbivorous insects (Dicke & Baldwin 2010; Hare 2011; Kessler & Heil 2011). On the one side, there is accumulating evidence that herbivore-damaged

plants may gain a fitness benefit from the recruitment of natural enemies (van Loon *et al.* 2000; Hoballah & Turlings 2001; Smallegange *et al.* 2008; Schuman *et al.* 2012b). On the other side, releasing volatiles may also cause plants to become apparent to herbivores that exert negative effects on plant fitness (Bruce *et al.* 2005; Halitschke *et al.* 2008). Our current study reveals that plant-derived volatiles can also attract enemies of beneficial insects. In addition, these hyperparasitoids show high specificity to HIPVs even under complex field conditions. Taking all into consideration, we notice that actually two out of three trophic levels of consumers (herbivorous insects and hyperparasitoids) that use plant volatiles for host location are unfavourable to the plants. Therefore, releasing HIPVs does not necessarily result in a fitness benefit to plants. From an evolutionary point of view, plants seem to be caught in a paradox whether natural selection favours plants that are "better emitter" at a cost of becoming apparent to every member in the community, or those that are "dumb" and draw less attention, without receiving the benefits of attraction of the third trophic level. Our result may help to improve our understanding of behavioural and community ecology of plants, beyond the "cry for help". Nevertheless, the fitness benefit of volatile emission still requires further evaluation in the context of the plant-associated insect food chain with the fourth trophic level organisms involved (Dicke & Baldwin 2010; Kaplan 2012). Plant breeding programs (to be included in Integrated Pest Management programs) that aim to enhance the production of HIPVs for natural enemy recruitment should also take unwanted attraction of unfavourable organisms into consideration (Kappers *et al.* 2010; Kappers *et al.* 2011).

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Chapter 4

Parasitism overrides herbivore identity allowing hyperparasitoids to locate their parasitoid host using herbivore-induced plant volatiles

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Abstract

Foraging success of predators profoundly depends on reliable and detectable cues indicating the presence of their often inconspicuous prey. Carnivorous insects rely on chemical cues to optimize foraging efficiency. Hyperparasitoids that lay their eggs in the larvae or pupae of parasitic wasps may find their parasitoid hosts developing in different herbivores. They can use herbivore-induced plant volatiles (HIPVs) to locate parasitized caterpillars. Because different herbivore species induce different HIPV emission from plants, hyperparasitoids may have to deal with large variation in volatile information that indicates host presence. In the current study, we used an ecogenomics approach to first address whether parasitized caterpillars of two herbivore species (*Pieris rapae* and *P. brassicae*) induce similar transcriptional and metabolomic responses in wild *Brassica oleracea* plants; and second, whether hyperparasitoids *Lysibia nana* are able to discriminate between these induced plant responses to locate their parasitoid host in different herbivores under both laboratory and field conditions. Our study revealed that both herbivore identity and parasitism affect plant transcriptional and metabolic responses to herbivory. We also found that hyperparasitoids are able to respond to HIPVs released by wild *B. oleracea* under both laboratory and field conditions. In addition, we observed stronger attraction of hyperparasitoids to HIPVs when plants were infested with parasitized caterpillars. However, hyperparasitoids were equally attracted to plants infested by either herbivore species. Our results indicate that parasitism plays a major role in HIPV-mediated plant-hyperparasitoid interactions. Furthermore, these findings also indicate that plant trait-mediated indirect interaction networks play important roles in community-wide species interactions.

Keywords: extended phenotype, herbivore-associated organism, herbivore-induced plant volatiles, hyperparasitoid, multi-trophic interactions, parasitism.

Introduction

In natural systems, resources exploited by a consumer are not necessarily distributed homogeneously, but instead may be heterogeneously embedded in much larger patches of resources that are nutritionally unsuitable. The ability of consumers to find and exploit suitable resources lies at the heart of optimal foraging theory (Pyke 1984). Specialist herbivores, for example, may need to explore their food plants that grow among a diverse range of non-food plants. In turn, natural enemies of these herbivores need to locate their prey in the often structurally complex vegetation and among assemblies of non-prey organisms (de Rijk *et al.* 2013). To optimize foraging, organisms often rely on cues that reliably predict the presence of their food source (Schoonhoven *et al.* 2005; Turlings *et al.* 2012). For insects in particular, various chemical cues offer a reliable and detectable source of information on the presence of a food plant or prey item (Godfray 1994; Bruce *et al.* 2005; Vet & Dicke 1992).

Among these chemical cues, plant volatiles have long been recognized as playing an important role in localizing food plants and prey by herbivorous and predatory insects, respectively. Recognition of food-plant odours by herbivores relies on either species-specific volatile compounds, or specific ratios of ubiquitous compounds (Visser 1986; Bruce *et al.* 2005; Webster 2012). Herbivorous insects may also detect changes in plant volatile profiles and use this information to determine whether the host plant has been colonised by other organisms (Fernandez & Hilker 2007; Poelman *et al.* 2008a; Stam *et al.* 2014).

In their turn, carnivorous insects, such as parasitic wasps, are able to use plant volatiles for host searching and these wasps can recognize specific plant volatile blends induced by their herbivore hosts (Dicke & Baldwin 2010). This indicates that plants have specific responses to herbivory depending on the identity of the herbivorous attacker. On the one hand, these finely-tuned induced plant responses can depend on the level of food plant specialization of the insect herbivores (Voelckel & Baldwin 2004; Diezel *et al.* 2009; Ali & Agrawal 2012). On the other hand, insect herbivores from different feeding guilds may also affect plant responses differently by inducing different signal-transduction pathways (De Vos *et al.* 2005; Broekgaarden *et al.* 2010a; Bidart-Bouzat & Kliebenstein 2011) that allow parasitoids to discriminate between volatiles induced by leaf chewing and phloem-feeding herbivores (van Poecke *et al.* 2003; de Rijk *et al.* 2013). In a complex natural environment, host searching by parasitoids may be hampered by the presence of different herbivore species on different food plants, or assemblies of herbivores that induce different odours in the same plant. Therefore, parasitoids may need to make the best use of the available volatile information to locate their hosts in species-rich environments (de Rijk *et al.* 2013).

For organisms towards the end of the food chain, such as hyperparasitoids, it may be even more challenging to locate their hosts. Primary hyperparasitoids parasitize the larvae of a parasitoid host while it is developing within the body of its herbivore host, whereas secondary hyperparasitoids attack the pre-pupae or pupae of their parasitoid host once the association has been terminated and can only accept newly formed pupae not older than two to three days (Sullivan 1987). To locate their parasitoid host, both primary and secondary hyperparasitoids use volatile information of plants induced by parasitized herbivores at long range and can discriminate between the body odours of parasitized caterpillars and healthy caterpillars at close range (Poelman *et al.* 2012; Zhu *et al.* 2014a; Zhu *et al.* 2014b). However, in nature, the parasitoid larvae may live in different herbivore host species that may induce different plant volatiles. Therefore, hyperparasitoids need to deal with a potentially large variation in odours that are associated with the presence of their hosts. Yet, whether hyperparasitoids exhibit preferences for plant volatile cues indicating the location of their host when it is developing inside different herbivore species is unknown.

In this study, we used a wild *Brassica oleracea* based food-web system including four trophic levels (Figure S1), to investigate whether hyperparasitoids are able to discriminate plant volatiles induced by different herbivore species carrying the same parasitoid. In our study system, *Lysibia nana* Granvenhost (Hymenoptera: Ichneumonidae) is an important secondary hyperparasitoid of the parasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae) (Harvey *et al.* 2003; Harvey 2008; Poelman *et al.* 2012; Poelman *et al.* 2013). *Lysibia. nana* uses herbivore-induced plant volatiles (HIPVs) emitted upon feeding by parasitized caterpillars as cue to locate parasitoid cocoons (Poelman *et al.* 2012). The variation in volatile emission by parasitized and unparasitized caterpillar feeding is most strongly driven by effects of parasitism on herbivore oral secretions and not by differences in feeding damage by parasitized and unparasitized caterpillars (Poelman *et al.* 2012). In the Netherlands, the larvae of the primary parasitoid *C. glomerata* are able to develop and are frequently found in two different *Pieris* caterpillars, *Pieris rapae* L. (Lepidoptera: Pieridae) and *P. brassicae* L. (Lepidoptera: Pieridae) that co-occur as specialist herbivore insects on *Brassica* plants. Larvae of *P. rapae* often occur as solitary individuals, whereas larvae of *P. brassicae* feed gregariously. Because of differences in their feeding behaviour and oral secretions, the two *Pieris* species may induce different responses while feeding on their food plant (Geervliet *et al.* 1998; Poelman *et al.* 2012). Thereby feeding by different parasitized caterpillars may also result in different responses in plants. Therefore, hyperparasitoids are expected to be able to exploit (variation in) plant cues induced by different parasitized caterpillars to maximize host-finding efficiency.

Using an integrated approach that includes transcriptomics, metabolomics, and insect behavioural assays, we specifically addressed the questions: 1) whether parasitized

caterpillars of the two *Pieris* species induce similar transcriptional and metabolomic plant responses; 2) whether these induced plant responses allow hyperparasitoids to locate their parasitoid host in different herbivores under both laboratory and field conditions.

Materials and Methods

Plants and insects

Seeds of the wild *Brassica oleracea* population "Kimmeridge" (Dorset, UK, 50°36′N, 2°07′W) were used. The *B. oleracea* plants used for all experiments (except for field assays) were grown in 2 liter pots containing peat soil (Lentse potgrond no. 4; Lent, The Netherlands) and provided with SON-T light (500 mmol/m2/s; L16:D8) in addition to natural daylight in a glasshouse compartment (22 ± 3 °C, 50-70% relative humidity, and 16:8 h L:D photoperiod). Five-week-old plants were used in the experiments. The Kimmeridge population is attacked by the two *Pieris* species and harbours a diverse array of herbivores in the field (Newton *et al.* 2010). Compared to other *B. oleracea* populations the Kimmeridge population is characterised by strong induced responses to *Pieris* herbivory and therefore selected for this study (Gols *et al.* 2008).

The herbivores (*Pieris rapae* and *P. brassicae*) and parasitoids (*Cotesia glomerata*) were originally collected from field sites near Wageningen University, The Netherlands and reared on cabbage plants (*B. oleracea* var *gemmifera* cv. Cyrus) in glasshouse compartments (22 ± 1 °C, 50-70% relative humidity, and 16:8 h L:D photoperiod). To prepare parasitized caterpillars for the induction treatments, individual first instar *P. rapae* or *P. brassicae* larvae were exposed to a single female *C. glomerata*, which was allowed to parasitize the caterpillar in a glass vial. The caterpillar was considered to be parasitized when the wasp had inserted her ovipositor in the caterpillar for at least 5 seconds. No more than ten caterpillars were offered to a single female to avoid effects caused by depletion of the parasitoids' egg load. The parasitized caterpillars were reared on cultivated *B. oleracea* plants until the fifth instar when they were used for induction treatments. The hyperparasitoid *Lysibia nana* was originally recovered from *C. glomerata* cocoons collected form field sites near Wageningen University, The Netherlands and was reared on *C. glomerata* cocoons in the absence of plant and herbivore-derived cues.

RNA extraction and microarray analysis

To characterize the transcriptional response of wild *B. oleracea* "Kimmeridge" plants after herbivory by unparasitized and parasitized caterpillars from two *Pieris* species, 5-week-old plants were treated with: 1) unparasitized *P. rapae* caterpillars (PR); 2)

unparasitized *P. brassicae* caterpillars (PB); 3) *P. rapae* caterpillars parasitized by *C. glomerata* (PR-CG), 4) *P. brassicae* caterpillars parasitized by *C. glomerata* (PB-CG) , or 5) were left untreated serving as the undamaged control (UD). All herbivore inductions were done with two fifth-instar larvae per plant. Twenty-four hours after infestation, we removed caterpillars and their frass, and subsequently collected one leaf disc (2.3 cm in diameter) from the first fully expanded and herbivore-damaged leaf of individual plants. Three biological replicates that each contained a pool of leaf disks from 20 plants were used for each treatment. Material was immediately flash frozen in liquid nitrogen after collection. Frozen leaf tissue was grinded and total RNA was isolated using the RNeasy Plant Mini Kit (QIAGEN, Venlo, The Netherlands), and then treated with RNase-free DNase Kit (QIAGEN) to remove genomic DNA. One microgram of total RNA from each sample was sent to the NASC Affymetrix Service (http://arabidopsis.info/) for hybridization to the Affymetrix *Brassica* Exon 1.0 ST GeneChip. In brief, the *Brassica* Exon 1.0 ST Array is single-colour based, representing 135,201 *Brassica* unigenes (Love *et al.* 2010), each unigene representing a unique expressed sequence tag (EST). The identifier for the annotation is GPL10733. The expression data were subjected to normalization using the Robust Multiarray Average (RMA) method from the Bioconductor software package (Gentleman *et al.* 2004). Log_2-transformed expression values were identified as differentially expressed using Student's *t*-test by comparing each herbivore treatment to the UD.

To further investigate the differentially experessed *Brassica* unigenes, their homologues in *Arabidopsis thaliana* were identified (http://www.*brassica*.info/). Arabidopsis gene descriptions and Gene Ontology (GO) annotations were obtained from TAIR (www.arabidopsis.org; TAIR genome v9, 20/07/2013). Identification and enrichment of GO terms within significantly differentially regulated sets of genes were obtained using the online tool provided by DAVID Bioinformatics Resources (http://david.abcc.ncifcrf.gov/). Venn diagrams and basic comparisons were made in Microsoft Excel.

Quantitative real-time PCR analysis

To confirm the results obtained in the genome-wide microarray analyses, qPCR analysis was performed on RNA isolated from plant material from a second, independent experiment. Plant induction, leaf-disc collections and RNA isolation followed the protocol described above. We collected five biological replicates that each comprised of a pool of leaf disks from 8 plants. cDNA was synthesized from 1 µg of RNA using an iScript cDNA synthesis kit (Bio-Rad, The Netherlands) in a 20 µl reaction volume. We selected seven genes that were induced by all treatments from the microarray experiment and the primer sequences used in this study are listed in supplementary Table S1. Primers were designed using Primer3Plus (http://www.bioinformatics.nl/primer3plus/) and were tested for specificity and efficiency before qPCR experiments.

Quantitative PCR was performed in Rotor-Gene Q (Qiagen, The Netherlands) in a total volume of 20 µl containing 5 ng of cDNA, 10 µl of iQ SYBR green supermix (Bio-Rad, The Netherlands), and 300 nmol/L of each gene-specific primer. The Ct values were normalized for differences in cDNA synthesis by subtracting the Ct value of the constitutively expressed gene *GAPDH* (glyceraldehyde-3-phosphate dehydrogenase) from the Ct value of the gene of interest (ΔCt). *GAPDH* has been proven to be a good housekeeping gene in *B. oleracea* (Zheng *et al.* 2007) and is frequently used as a reference gene in expression studies (Carraro *et al.* 2005; Broekgaarden *et al.* 2010a). Relative gene expression ($2^{-\Delta\Delta Ct}$) was calculated according to Livak and Schmittgen (2001) and (Pfaffl 2001).

Headspace collection of plant volatiles

To characterize the effects of herbivore identity and parasitism on the volatile emission by wild *B. oleracea*, we treated 5-week-old plants in the same way as described for plant transcriptome analysis. Shorly before volatile collections, we removed the caterpillars and their frass from plants. Dynamic headspace sampling was carried out in a climate room, and we collected 10 replicates from each of the five experimental treatments (UD, PR, PB, PR-CG, and PB-CG). Pots were carefully wrapped in aluminium foil to minimize odour contribution from pots and/or soil. During volatile collection, the plants were placed individually into a 30 litre glass jar, which was sealed with a viton-lined glass lid with an inlet and outlet. Compressed air was filtered by passing through charcoal before reaching the glass jar containing the plant. Volatiles were collected by sucking air out of the glass jar at a rate of 200 ml/min through a stainless steel tube filled with 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) for 2 h.

Analysis of plant volatiles

A combination of Thermo Trace Ultra gas chromatography (GC) and Thermo Trace DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA) was used for the analysis of volatiles associated with plants induced by unparasitized or parasitized caterpillars from the two *Pieris* species. Prior to releasing the volatiles, each sample was dry-purged under a stream of nitrogen (50 ml/min) for 10 min at room temperature (21 ± 2 °C) in order to remove moisture. The volatiles were then thermally released from the Tenax TA (CAMSCO) using an Ultra 50:50 thermal desorption unit (Markes, Llantrisant, UK) at 250 °C for 10 min under a helium flow of 20 ml/min, while re-collecting the volatiles in a cooled solvent trap – Unity (Markes) at 10 °C. Once the desorption process was completed, volatiles were released from the cold trap by fast heating at 40 °C/s to 280 °C, which was then kept for 10 min, while the volatiles were transferred to a ZB-5MSi analytical column [30 m L x 0.25 mm I.D. x 1.00 µm F.T. (Phenomenex, Torrance, CA, USA)], in a splitless mode for further separation. The GC was operated at an initial oven temperature of 40 °C and was

immediately raised at 5 °C/min to a final temperature of 280 °C, where it was held for 4 min under a helium flow of 1 ml/min in a constant flow mode. The DSQ mass spectrometer (MS) was run in a scan mode in a mass range of 35 - 400 amu at 4.70 scans per second and mass spectra were recorded in electron impact ionisation (EI) mode at 70 eV. The temperatures of the MS transfer line and ion source were set to 275 and 250 °C, respectively. Tentative identification of compounds was based on comparison of mass spectra and linear retention indices (LRI) with those in the NIST 2005 and Wageningen Mass Spectral Database of Natural Products mass spectra libraries. We analysed all samples and reference alkanes for the RI in a full scan mode under the same analytical conditions and total ion current (TIC) chromatograms were obtained. A target (single) ion for each compound was used for the measurement of peak area. Volatiles collected from compressed air, empty glass jars, clean Tenax TA adsorbents including those sourced from the analytical system itself were treated as blank samples and used for corrective measures during analysis.

Y-Tube olfactometer assays

To test whether herbivore identities may influence behavioural responses of *L. nana* to plant volatiles, we offered *L. nana* females two choices for combinations among the five treatments (UD, PR, PB, PR-CG, and PB-CG) in Y-tube olfactometer assays, following the same protocol of 24h induction by two caterpillars as in the transcript and volatiles analysis. First, we tested preferences of *L. nana* to plant volatiles emitted by UD versus all other four herbivory treatments, to assess the attraction of hyperparasitoids to HIPVs released by wild *B. oleracea*. Then, we assessed preferences of *L. nana* to plant volatiles induced by unparasitized and parasitized caterpillars within herbivore species. Finally, we studied the attactiveness of HIPVs across herbivore species to the hyperparasitoids.

Shortly before *L. nana* females were tested for their behavioural response to plant volatiles in two choice Y-tube olfactometer bio-assays, we removed caterpillars and their feces from the plants. The plants were placed in one of two glass jars (30 L each) that were connected to the two olfactometer arms. A charcoal-filtered airflow (3 L/min) was led through each arm of the Y-tube olfactometer system, and a single naive wasp was released at the base of the stem section (3.5 cm diameter, 22 cm length) in each test. Wasps that reached the end of one of the olfactometer arms within 10 min and stayed there for at least 10 s were considered to have chosen the odour source connected to that olfactometer arm. We swapped the jars containing the plants after testing five wasps, in order to compensate for unforeseen asymmetry in the setup. Each set of plants was tested for 10 wasps and seven sets of plants for each combination were tested. After each set of plants was tested, the glass jars were cleaned using distilled water and dried with tissue paper. The Y-tube olfactometer

setup was placed in a climatized room, and in addition to daylight it was illuminated with four fluorescent tube lights (FTD 32 W/84 HF, Pope, the Netherlands).

Field assay

Fifty four-week-old plants (grown in glasshouse compartment) were transplanted into the field with 1x1 m spacing between plants and allowed to adjust to field conditions for one week. Thereafter, the plants were subjected to the same five herbivore inductions as in the Y-tube olfactometer assays. However, unparasitized and parasitized first-instar caterpillars were allowed to feed on plants for 10 d, which was approximately the whole developmental period of *C. glomerata* larvae within their caterpillar host. When transplanted to the field, each plant was covered with a fine-mesh net to avoid other herbivore infestations on the foliage and to prevent the herbivores used for induction to wander off the plant.

To test the effects of plant induction by different types of herbivory on hyperparasitism, we attached *C. glomerata* cocoon clutches onto the plants in the field. Individual cocoon clutches of *C. glomerata* were first attached to a paper disc (3x3 cm) with a small droplet of glue (HEMA, the Netherlands). We removed nets and caterpillars just before attaching the paper discs carrying the parasitoid pupae with a pin. We attached five cocoon clutches onto each plant. The cocoons were exposed to the natural community of hyperparasitoids and recollected after 5 d. Subsequently, they were kept in the laboratory separately in 2 ml Eppendorf tubes that were closed with cotton wool. The Eppendorf tubes were checked daily for emerging primary parasitoids and hyperparasitoids. All wasps were identified to species level.

A completely randomized design was applied to the field assays. We repeated the experiment four times from July until October in two field seasons (2012 and 2013) each using 50 plants that included 10 replicates of each treatment.

Statistical analysis

Both the gene expression and volatile emission multivariate data analysis were carried-out using projection to latent structures-discriminant analysis (PLS-DA). For gene expression analysis, the measured gene expression levels that were significantly different between undamaged control and all other herbivore treatments were log-transformed, mean-centred and scaled to unit variance before being analysed using PLS-DA. The results of the analysis are visualized in score plots, which reveal the sample structure according to the model components. For volatile analysis, the measured peak area for the volatile blends in the different treatments were log-transformed, mean-centred and scaled to unit variance before being analysed using PLS-DA. The results of the analysis are visualized in score plots and loading plots. The score plots reveal the sample structure according to the model components. The loading plots display the contribution

of the variables to the components and the relationships among the variables. Mann-Whitney U tests were used to test the differences in emission of individual volatile compounds that were tentatively identified in the headspace of wild *B. oleracea* plants.

Lysibia nana preferences for HIPVs were analysed using two-tailed binomial tests.

The differences in hyperparasitism ratio under field condition among plant induction treatments were analysed using two Generalized Linear Models (GLMs). In the first model, to analyse the effects of plant inductions with different types of herbivory on hyperparasitism ratio, we modelled the dependent variable as the number of clutches giving any hyperparasitoid out of the fixed totals of five cluthes attached to the plant. We included caterpillar induction treatment (UD, PR, PB, PR-CG, or PB-CG) as fixed factor. In the second model, we included the fixed factor herbivore species (*P. rapae* or *P. brassicae*), parasitism (parasitized or unparasitized) and their interaction to evaluate the overall effect of parasitism and herbivore identity on hyperparasitism.

All statistical analyses were performed with the statistical software package IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA), except the multivariate data analysis (PLS-DA), which was carried out using the SIMCA P+ version 12.0.1.0 (Umetrics, Umeå, Sweden).

Results

Plant gene expression changes in response to feeding by unparasitized or parasitized caterpillars from two *Pieris* species

Feeding by unparasitized *P. rapae* or *P. brassicae* caterpillars resulted in a total of 2763 and 4041 differentially expressed *Brassica* unigenes (2-fold or greater; false discovery rate (FDR) $P < 0.05$), respectively, compared to undamaged control plants (Figure 1). Plants infested with *P. rapae* or *P. brassicae* caterpillars that were parasitized by *C. glomerata* showed 3278 and 4069 differentially expressed unigenes compared to undamaged control plants, respectively (Figure 1). A projection to latent structures-discriminant analysis (PLS-DA) using expression levels of all differentially expressed unigenes showed clear separation between the four herbivore treatments and undamaged control, as well as among the four herbivore treatments (Figure 2). By direct comparisons of plant gene expression levels among different herbivore treatments (PR vs PB; PR-CG vs PB-CG; PR vs PR-CG; PB vs PB-CG), we found that 73 *Brassica* unigenes were differentially regulated in *C. glomerata*-parasitized *P. rapae* compared to unparasitized *P. rapae* (PR-CG vs PR) treated plants (Table S2). We also found 31 *Brassica* unigenes were differentially regulated in *C. glomerata*-parasitized *P. rapae* compared to *C. glomerata*-parasitized *P. brassicae* (PR-CG vs PB-CG) treated plants (Table S3). With regard to the effects of herbivore species on plant induction, 157

Brassica unigenes were differerially regulated in *P. rapae* (PR and/or PR-CG) compared to P, *brassica*e (PB and/or PB-CG) induced plants (Table S4). Furthermore, there were 17 *Brassica* unigenes differerially regulated comparing unparasitized (PR or PB) and *C. glomerata* parasitized caterpillars (PR-CG or PB-CG) induced plants (Table S5).

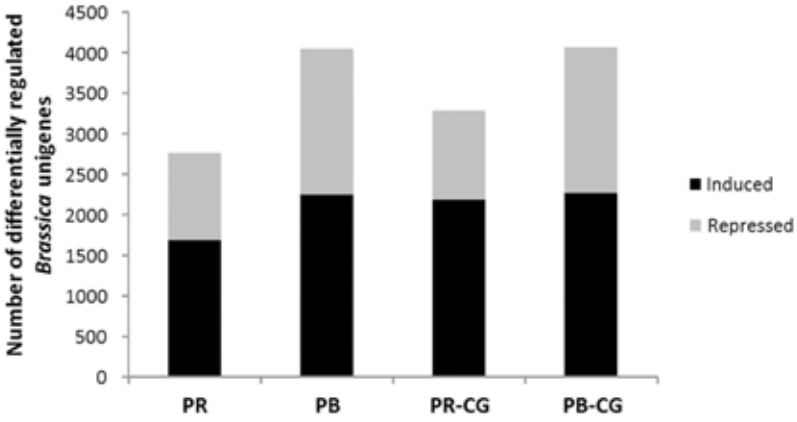

Figure 1. Transcriptional responses of wild *Brassica oleracea* plants to insect infestation. The number of *Brassica* unigenes that were significantly induced (black bars) or repressed (gray bars) in plants after infestation by unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *Cotesia glomerata*-parasitized *P. rapae* (PR-CG), or *C. glomerata*-parasitized *P. brassicae* (PB-CG) compared to undamaged control plants (Student's t tests, FDR, $P < 0.05$; fold-change > 2).

Gene ontology-enrichment analysis using *A. thaliana* homologues of up-regulated unigenes induced by different herbivore treatments resulted in 50 significantly enriched functional categories related to biological processes. Genes involved in defense, stress response, metal ion transport, secondary metabolism, and JA signaling, were overrepresented in all herbivore inductions (Figure S2). Parasitized caterpillars induced more genes involved in glucosinolate bio-synthetic processes in plants than unparasitized caterpillars (Figure S2). Plants treated with either unparasitized or parasitized *P. brassicae* caterpillars showed significant up-regulation of genes involved in response to bacteria and in fatty acid metabolic processes (Figure S2). The genes that were down-regulated represented 18 significantly enriched functional categories, including photosynthesis and responses to the abiotic stress factors temperature and light (Figure S3). Remarkably, with regard to the homologues in *Arabidopsis* of up-regulated *Brassica* unigenes in response to different herbivore feeding treatments, herbivore species as well as parasitism showed effects on plant transcriptional responses, but also shared a large overlap (Figure 3). The repressed *Brassica* unigenes caused by different treatments also showed similarity and specificity to each treatment (Figure 3).

Seven randomly selected genes that were investigated for microarray validation showed similar expression patterns among the five treatments in the RT-qPCR and microarray analyses (Figure S4), indicating the reliability of the microarray data.

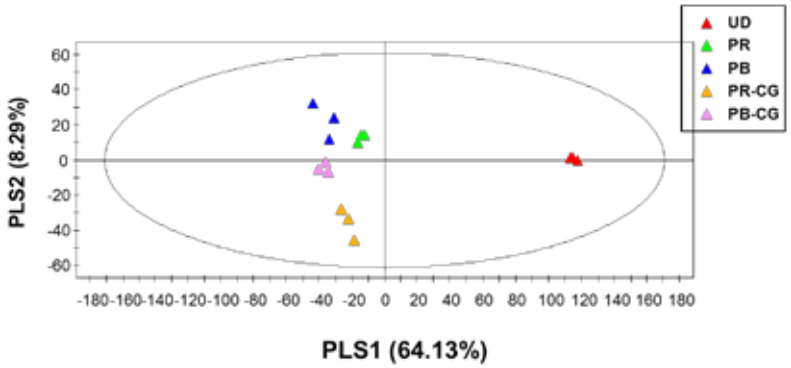

Figure 2. Projection to Latent Structures-Discriminant Analysis (PLS-DA) of gene expression levels in plants that were damaged by either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *Cotesia glomerata*-parasitized *P. rapae* (PR-CG), *C. glomerata*-parasitized *P. brassicae* (PB-CG), or remained intact as undamaged control (UD), for the 5585 genes that displayed significant differences (FDR, P < 0.05) between each herbivore treatment and the undamaged control. The score plot visualizes the structure of the samples according to the first two principal components with the explained variance in brackets. The ellipse defines the Hotelling's T2 confidence region (95%).

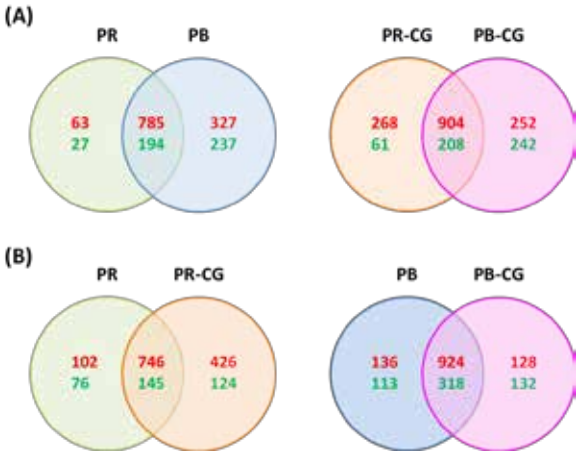

Figure 3. Transcriptional changes in wild *Brassica oleracea* plants induced by either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *Cotesia glomerata*-parasitized *P. rapae* (PR-CG), or *C. glomerata*-parasitized *P. brassicae* (PB-CG). The numbers indicate the total number of induced (red) or repressed (green) homologues in *Arabidopsis thaliana* corresponding (A) to each herbivory treatment, or (B) to direct comparisons between induction by unparasitized or parasitized caterpillars from two *Pieris* species.

Plant volatile analysis

Analysis of the volatile blends of wild *B. oleracea* plants induced by *P. rapae*, *P. brassicae*, *C. glomerata* parasitized *P. rapae* or *P. brassicae* revealed 44 compounds that were present in at least 50% of all samples. These compounds were tentatively identified (Table 1), their measured peak areas were corrected for above ground fresh weight of each corresponding plant sample and used for further analysis. PLS-DA analysis of the volatile blends showed that all four herbivore treatments induced volatile blends that

differed from undamaged control plants (Figure 4A). Among these four herbivore damage treatments, plants damaged by unparasitized *P. rapae* caterpillars were less than 20% similar in their volatile headspace to plant headspaces induced by unparasitized *P. brassicae* caterpillars. However, plants induced by parasitized *P. rapae* or *P. brassicae* caterpillars overlapped more than 50% in their volatile headspace composition as shown by PLS-DA and differed from volatile blends induced by unparasitized caterpillars (less than 50% overlap). Sixteen compounds contributed most strongly to the differences among treatments as indicated by Variable Importance in the Projection (VIP) scores being higher than 1 (Table 1). These compounds include terpenoids, aliphatic and aromatic carbonyls, nitriles and green-leaf volatiles. A nitrile (2,4-penta-dienenitrile) was closely associated with parasitized caterpillar treated plants (Figure 4B). Several compounds, including terpenoids, nitriles, ketones and various green-leaf volatiles, were associated with unparasitized *P. rapae* or *P. brassicae* induced plants (Figure 4B).

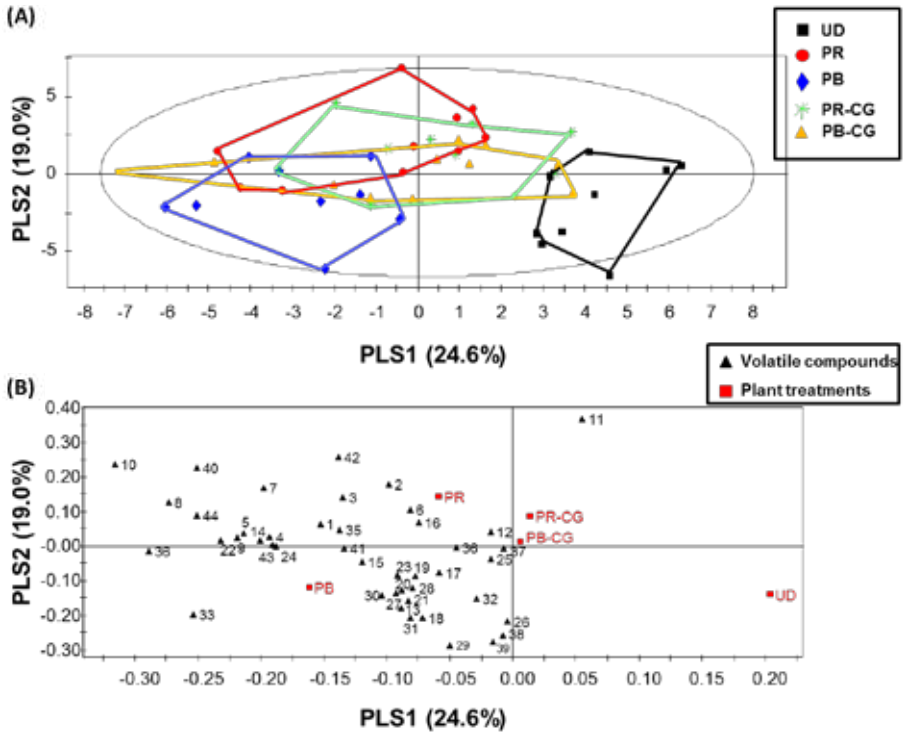

Figure 4. Projection to Latent Structures-Discriminant Analysis (PLS-DA) of the blends of volatile compounds emitted by plants in response to either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *Cotesia glomerata*-parasitized *P. rapae* (PR-CG), or *C. glomerata*-parasitized *P. brassicae* caterpillars (PB-CG), or plants that had remained undamaged (UD). The score plot (A) visualizes the structure of the samples according to the first two principal components with the explained variance in brackets. The Hotelling's T2 ellipse confines the confidence region (95%) of the score plot. The loading plot (B) defines the contribution of each of the volatile compounds to the first two principal components. Compound identities and their respective numbers are presented in Table 1.

Responses of hyperparasitoids to plant volatiles induced by two species of parasitized herbivores

Approximately 70% of the hyperparasitoids made a choice in the Y-tube olfactometer experiments. The wasps preferred plant volatiles induced by all herbivore treatments (unparasitized *P. rapae* or *P. brassicae*, as well as *P. rapae* or *P. brassicae* that were parasitized by *C. glomerata*) over undamaged plants (Figure 5; binomial tests, $P < 0.01$). For both *Pieris* species, plant volatiles induced by *C. glomerata*–parasitized caterpillars were more attractive to *L. nana* than volatiles from plants damaged by unparasitized caterpillars (Figure 5; binomial tests, $P < 0.01$). The hyperparasitoids showed equal preferences to plant volatiles induced by the two herbivore species when they were unparasitized (PR vs PB) or parasitized (PR-CG vs PB-CG) by *C. glomerata* (Figure 5; binomial tests, $P = 0.67$ and $P = 0.68$, respectively).

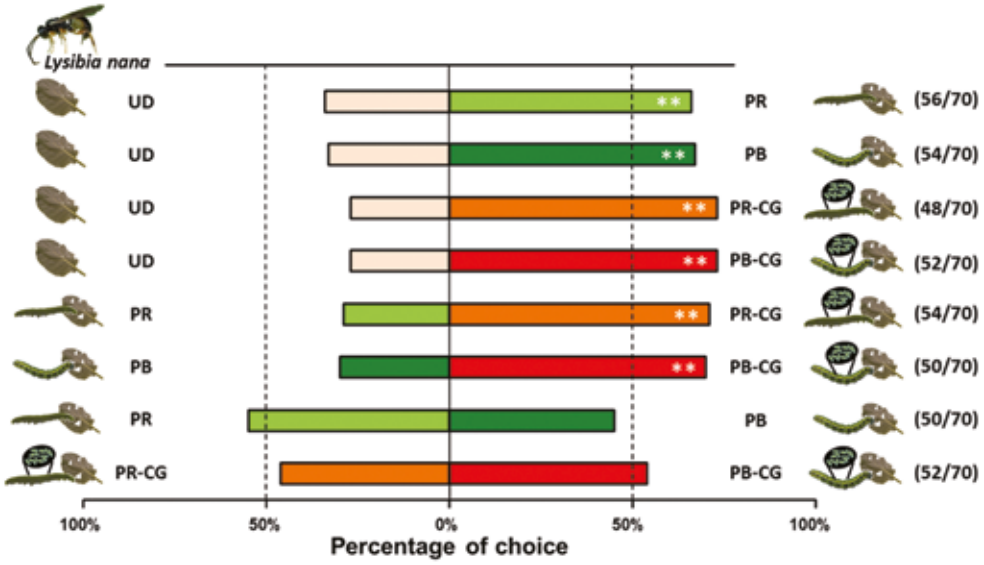

Figure 5. Preference of hyperparasitoids for herbivore-induced plant volatiles in two choice Y-tube olfactometer tests, comparing undamaged control plants (UD), *Pieris rapae*-damaged plants (PR), *P. brassicae*-damaged plants (PB), plants damaged by *Cotesia glomerata*-parasitized *P. rapae* caterpillars (PR-CG), and plants damaged by *C. glomerata*-parasitized *P. brassicae* caterpillars (PB-CG). Numbers between brackets indicate the number of wasps that made a choice within 10 min from the start of the experiment versus the total number of wasps tested. **: $P < 0.01$.

Table 1. Volatile compounds, tentatively identified in the headspace of wild *Brassica oleracea* "Kimmeridge" plants. Volatile emissions are given as mean peak area (SE) per gram fresh weight of plant divided by 10^3.Variable Importance in the Projection (VIP) values for the PLS-DA are given. VIP values larger than 1 are shown boldfaced. Differences among treatments for compounds based on Mann–Whitney U pair wise comparisons are indicated with superscript letters.

No.	Compound	Class	UD^x (n = 9)	PR^x (n = 10)	PB^x (n = 9)	PR-CG^x (n = 10)	PB-CG^x (n = 10)	VIP score
1	2-methylpropanenitrile	Nitrile	22.18 (2.10)^a	54.21 (16.64)^b	91.49 (42.49)^b	51.18 (11.81)^b	64.18 (33.58)^b	0.81
2	(*E*)-2-butenenitrile	Nitrile	0.00 (0.00)^a	43.67 (9.27)^c	28.86 (3.86)^{bc}	30.89 (5.46)^{bc}	20.78 (5.37)^b	0.86
3	3-butenenitrile	Nitrile	0.00 (0.00)^a	128.33 (20.56)^c	92.89 (14.44)^{bc}	87.38 (22.26)^{bc}	69.20 (20.60)^b	0.87
4	1-penten-3-ol	Alcohol	51.17 (24.14)^a	248.43 (103.80)^b	398.88 (161.30)^b	221.30 (136.17)^b	218.04 (102.59)^b	0.99
5	3-pentanone	Ketone	4.96 (1.29)^a	23.58 (10.44)^b	42.39 (15.99)^b	28.11 (15.17)^b	23.45 (11.44)^b	**1.1**
6	methylthiocyanate	Thiocyanate	37.23 (9.10)	50.73 (6.16)	38.26 (5.35)	39.12 (5.83)	40.06 (5.34)	0.58
7	2-methylbutanenitrile	Nitrile	8.49 (1.24)^a	240.49 (106.87)^b	588.45 (307.67)^b	245.49 (81.69)^b	405.09 (332.04)^b	**1.18**
8	3-methylbutanenitrile	Nitrile	17.62 (4.39)^a	104.67 (16.49)^b	200.57 (99.50)^b	81.80 (20.97)^b	131.19 (87.45)^b	**1.46**
9	dimethyl disulfide	Sulfide	125.56 (20.26)^a	312.72 (92.84)^b	433.10 (79.06)^c	252.42 (79.46)^{ab}	353.73 (78.68)^{bc}	**1.13**
10	3-methyl-2-pentanone	Ketone	1.09 (0.37)^a	15.06 (3.66)^b	14.49 (2.85)^b	11.75 (2.99)^b	10.95 (2.97)^b	**1.83**
11	2,4-pentadienenitrile	Nitrile	0.56 (0.56)^a	52.50 (13.68)^c	9.47 (0.94)^b	36.44 (9.29)^c	6.25 (1.66)^b	**1.59**
12	(*E*)-2-hexenal	Aldehyde	10.17 (3.44)	10.02 (0.99)	8.28 (1.30)	9.30 (1.27)	7.92 (0.88)	0.19
13	unknown	Unknown	93.39 (20.59)^{ab}	90.83 (17.61)^{ab}	124.58 (17.20)^a	78.41 (8.35)^{ab}	72.71 (10.62)^b	0.91
14	(*Z*)-3-hexen-1-ol	Alcohol	28.74 (15.44)^a	245.80 (139.54)^b	301.03 (127.61)^b	79.10 (39.53)^b	107.56 (47.07)^b	**1.03**
15	(*Z*)-2-penten-1-ol acetate	Ester	4.92 (4.46)^a	83.11 (48.14)^b	188.94 (89.40)^b	40.07 (25.92)^b	60.02 (39.86)^b	0.66
16	butyrolactone	Ketone	69.16 (7.65)	79.88 (3.58)	74.80 (6.00)	72.06 (5.94)	71.84 (7.69)	0.46
17	α-thujene	Terpenoid	32.89 (14.83)	28.63 (9.72)	67.62 (23.80)	29.79 (10.12)	24.01 (7.26)	0.46
18	α-pinene	Terpenoid	18.38 (7.32)	17.32 (5.21)	38.79 (12.68)	20.13 (6.29)	14.66 (4.09)	0.97
19	sabinene	Terpenoid	143.86 (62.74)	139.33 (48.02)	320.41 (108.71)	132.33 (48.40)	117.39 (35.07)	0.57
20	β-pinene	Terpenoid	96.61 (31.23)	91.56 (23.79)	179.03 (53.18)	91.40 (24.67)	81.17 (16.88)	0.73
21	β-myrcene	Terpenoid	223.72 (93.54)	199.56(66.69)	453.99 (153.05)	185.82 (65.61)	171.96 (48.97)	0.82
22	(*Z*)-3-hexen-1-ol acetate	Ester	276.00 (201.24)^a	4124.05 (2447.28)^b	7006.11 (3189.69)^b	1021.63 (544.21)^b	2252.73 (1355.31)^b	**1.2**

No.	Compound	Class	UD[x] (n = 9)	PR[x] (n = 10)	PB[x] (n = 9)	PR-CG[x] (n = 10)	PB-CG[x] (n = 10)	VIP score
23	α-phellandrene	Terpenoid	11.19 (5.05)	8.81 (3.01)	19.45 (6.52)	7.85 (2.54)	6.70 (2.04)	0.62
24	hexyl acetate	Ester	7.78 (4.74)[a]	117.02 (67.64)[b]	198.12 (87.18)[b]	44.47 (18.82)[b]	68.69 (35.72)[b]	0.97
25	3-carene	Terpenoid	7.95 (1.15)	7.19 (1.36)	9.04 (1.99)	10.29 (2.46)	5.40 (0.82)	0.2
26	α-terpinene	Terpenoid	41.74 (20.49)	27.50 (9.34)	66.29 (23.62)	26.28 (8.73)	21.19 (6.76)	0.91
27	limonene	Terpenoid	189.54 (73.13)	172.81 (54.87)	353.18 (101.13)	174.57 (49.15)	137.22 (35.84)	0.77
28	1,8-cineole	Terpenoid	153.54 (56.22)	126.83 (42.17)	300.29 (104.88)	119.13 (38.98)	103.77 (28.88)	0.68
29	2-phenylacetaldehyde	Aldehyde	42.43 (3.71)[a]	35.92 (4.16)[a]	50.99 (4.54)[a]	33.33 (4.44)[a]	37.13 (2.61)[a]	**1.25**
30	(*E*)-β-ocimene	Terpenoid	8.78 (2.89)	9.66 (2.54)	17.78 (4.83)	7.46 (2.58)	6.90 (1.55)	0.84
31	γ-terpinene	Terpenoid	27.67 (12.60)	17.57 (5.80)	40.99 (14.40)	16.57 (5.32)	13.42 (4.28)	0.99
32	α-terpinolene	Terpenoid	19.97 (9.24)	14.64 (4.98)	34.66 (12.17)	12.84 (4.66)	11.34 (3.53)	0.66
33	(*E*)-DMNT	Terpenoid	6.56 (3.62)[a]	28.13 (9.24)[b]	100.28 (34.21)[b]	22.73 (8.09)[ab]	45.84 (12.02)[b]	**1.61**
34	phenylethyl alcohol	Alcohol	7.47 (0.51)[a]	23.97 (3.60)[bc]	59.11 (12.05)[c]	20.90 (6.29)[ab]	36.64 (14.19)[bc]	**1.5**
35	α-isophoron	Ketone	18.89 (2.37)	31.84 (5.84)	38.48 (8.27)	39.20 (15.03)	28.94 (4.91)	0.72
36	menthone	Terpenoid	80.54 (54.11)	64.04 (31.58)	57.95 (36.41)	3.35 (0.52)	31.97 (30.17)	0.23
37	menthol	Terpenoid	375.42 (240.92)[b]	454.54 (215.21)[b]	307.88 (193.34)[ab]	12.96 (1.45)[a]	191.69 (176.97)[ab]	0.04
38	terpinen-4-ol	Terpenoid	2.06 (0.80)[ab]	1.42 (0.50)[ab]	3.35 (1.23)[b]	1.16 (0.40)[ab]	0.69 (0.29)[a]	**1.08**
39	α-terpineol	Terpenoid	12.97 (4.77)[a]	4.65 (1.28)[a]	30.21 (12.67)[a]	5.25 (2.31)[a]	6.46 (2.42)[a]	**1.15**
40	indole	Hetrocyclic	6.93 (2.92)[a]	150.24 (36.47)[b]	125.09 (43.66)[b]	180.14 (108.53)[b]	86.18 (14.89)[b]	**1.53**
41	longifolene	Terpenoid	10.43 (0.88)[a]	13.00 (1.13)[ab]	19.05 (5.89)[b]	15.86 (4.02)[ab]	12.80 (1.86)[ab]	0.7
42	α-muurolene	Terpenoid	8.76 (3.80)[a]	36.15 (15.57)[a]	10.58 (4.43)[a]	5.89 (3.73)[a]	14.42 (3.77)[a]	**1.23**
43	(*E,E*)-alpha-farnesene	Terpenoid	18.47 (7.61)[a]	35.10 (9.39)[ab]	103.13 (51.48)[b]	35.80 (12.18)[ab]	27.00 (9.66)[ab]	0.99
44	(*E*)-nerolidol	Terpenoid	2.10 (1.15)[a]	16.97 (4.10)[b]	30.12 (6.11)[b]	15.64 (5.26)[b]	17.59 (3.42)[b]	**1.32**

[x]: Treatments that plants were subjected to: UD = undamaged control; PR = *Pieris rapae*; PB = *P. brassicae*; PR-CG = *Cotesia glomerata* parasitized *P. rapae*; PB-CG = *C. glomerata* parasitized *P. brassicae.*

Field assays

From *C. glomerata* cocoons in our field experiment, we recovered 5 species of hyperparasitoids of which *L. nana* and *Acrolyta nens* were the most abundant (Figure S5). *Cotesia glomerata* cocoons attached to herbivore-induced plants had a higher degree of hyperparasitism than those attached to undamaged control plants (Figure 6, Table 2). Herbivore species identity did not affect the hyperparasitism ratios. However, interestingly, *C. glomerata* cocoons on plants induced by parasitized herbivores received higher hyperparasitism ratio than those on plants induced by unparasitized herbivores (Figure 6, Table 3).

Table 2. The effect of plant induction treatment on the fraction of primary parasitoid cocoons per plant that contained any hyperparasitoid in the field.

Model factor	Deviance	Degrees of Freedom	p Value
Full model	352,602	437	
Factor			
Caterpillar induction		4	0.001
Replicate		8	0.009
Year		1	0.001

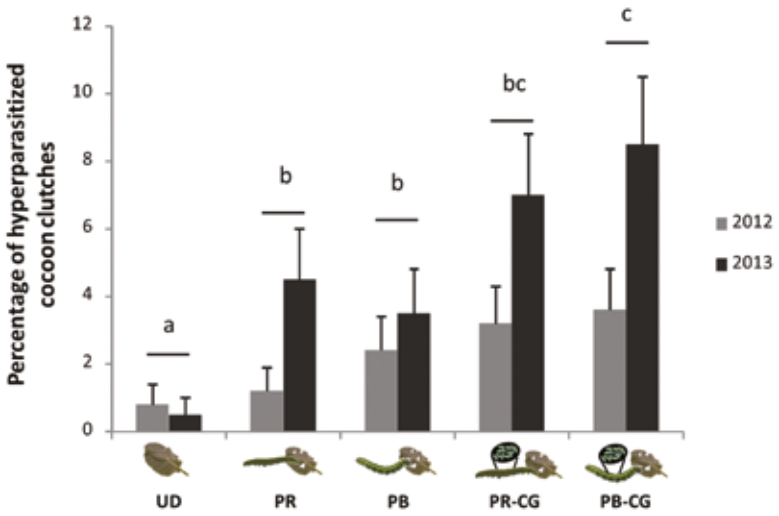

Figure 6. Percentage of *Cotesia glomerata* cocoon clutches that contained hyperparasitoids in the field trials of 2012 (gray bar) and 2013 (black bar). The cocoons were collected from plants that had previously been infested with *Pieris rapae* (PR), *Pieris brassicae* (PB), *Cotesia glomerata*-parasitized *P. rapae* (PR-CG) or *P. brassicae* (PB-CG), or plants that had previously remained undamaged (UD). Letters indicate significant differences between treatment groups (GLM, $p < 0.05$).

Table 3. The effect of herbivore identity and parasitism on the fraction of primary parasitoid cocoons per plant that contained any hyperparasitoid in the field.

Model factor	Deviance	Degrees of Freedom	p Value
Full model	353	357	
Factor			
Herbivore identity		1	0.55
Parasitism		1	0.007

Discussion

The foraging success of an organism largely depends on its ability to utilize reliable information indicating the presence of suitable food. To locate concealed prey, predators may have to rely on information that indirectly indicates their presence (Vet & Dicke 1992). Organisms in the fourth trophic level, such as hyperparasitoids, have to overcome a double-edged constraint in that their primary parasitoid hosts are developing inside the body of a herbivore host which may in turn be feeding from an inconspicuous part of its food plant (Sullivan & Volkl 1999). Hyperparasitoids use HIPVs to locate their hosts (Poelman *et al.* 2012). They may also differentiate between the volatiles induced or released by unparasitized herbivores or by herbivores carrying their primary parasitoid hosts (Poelman *et al.* 2012; Zhu *et al.* 2014b). Our current study shows that herbivore species as well as parasitism affect transcriptional and metabolomic plant responses to herbivory. In addition, although different herbivore species induced different plant responses, hyperparasitoids still were able to exploit HIPVs released by wild *B. oleracea* plants in response to caterpillars that are parasitized to locate their parasitoid hosts under both laboratory and field conditions.

Feeding by herbivorous insects induces plant transcriptional changes, thereby activating a suite of defence responses (Kessler & Baldwin 2002; van Dam 2009; Dicke & Baldwin 2010; Bonaventure 2012). These induced responses in plants may be greatly influenced by the feeding guilds and the level of food plant specialization of the herbivore attackers (Voelckel & Baldwin 2004; Bidart-Bouzat & Kliebenstein 2011; Ali & Agrawal 2012). However, plants induced by specialist or generalist herbivores from the same feeding guild may show large overlap with conserved transcript pattern (i.e. defense-related pathways) in microarray analysis (Reymond *et al.* 2004; Bidart-Bouzat & Kliebenstein 2011). In this study, we have investigated plant transcriptional changes induced by two specialist chewing *Pieris* herbivores. Our trancriptomics analysis reflected the chewing feeding features of these two herbivores (Browse & Howe 2008; Bari & Jones 2009). In response to *Pieris* caterpillar attack, plants activate defence-related genes involved in wound responses and jasmonic acid signaling (Reymond *et al.* 2000; Reymond *et al.* 2004; De Vos *et al.* 2005). We also

found that genes related to secondary metabolism, such as glucosinolate metabolic processes, were induced in response to chewing herbivore feeding (Reymond *et al.* 2004; Broekgaarden *et al.* 2007). In addition, photosynthesis-related genes were down-regulated in response to all herbivore treatments, which may be interpreted as reallocation of resources from phytosynthesis to the costly defences (Hermsmeier *et al.* 2001; Broekgaarden *et al.* 2011). However, the two closely-related *Pieris* species with similar feeding behaviours also induce different transcriptional changes in the plants either when the caterpillars are unparasitized or parasitized (Figures 2 & 3) (Poelman *et al.* 2011b).

In addition, the differences in plant responses to herbivory by different herbivore species were also apparent in the composition of the induced blend of plant volatiles. HIPVs induced by unparasitized *P. rapae* or *P. brassicae* were clearly different (Figure 4). Besides herbivore specialization and feeding guild, a wide range of herbivore-associated organisms (HAO) that develop in or on the herbivore, including bacteria (Chung *et al.* 2013), and viruses (Luan *et al.* 2013), may also affect plant responses to herbivory (Zhu *et al.* 2014a). Here, we found that parasitism of the herbivore by parasitoid larvae affected both transcriptomic and metabolomic responses of the plants (Figures 2-4). The parasitoid larvae physiologically manipulate their host species to optimize their own development. However, a consequence is that their presence becomes apparent by the effects on the emission of plant odours that hyperparasitoids may use to locate their parasitoid hosts (Poelman *et al.* 2012). Interestingly, plant volatile profiles induced by *P. rapae* or *P. brassicae* caterpillars that were parasitized by *C. glomerata* showed larger overlap compared to those induced by unparasitized caterpillars, suggesting that parasitoid larvae regulating their host cause considerable changes in their host that indirectly affect plant responses beyond variation in responses that healthy caterpillars of the two species induce. Therefore, parasitism overrides the effects of herbivore identity on the emission of induced volatiles in *B. oleracea* plants. The hyperparasitoids located their parasitoid host regardless of the caterpillar species they were developing in, which allows them to maximize foraging efficiency and consequently fitness. In this study, we sampled plant materials for transcriptomic experiments only after 24h of herbivory. Future studies should include time points from different induction phases to further improve our understanding of how plants cope with attack by unparasitized and parasitized herbivores.

Herbivore-induced plant responses profoundly affect the biodiversity of the insect community (Kessler *et al.* 2004; Poelman *et al.* 2008b; Poelman *et al.* 2010). Particularly, plant volatiles induced by herbivorous insects make the status of the plant apparent to subsequent colonizing herbivores and predators at higher trophic levels (Heil 2008; Dicke & Baldwin 2010; Mumm & Dicke 2010; Poelman *et al.* 2011b; Poelman *et al.* 2012). In our field experiments, we observed a strong year effect on

hyperparasitism rate (Figure 6; Table 2; Figure S5). Furthermore, we recorded higher levels of hyperparasitism for cocoons on plants that had previously been infested by herbivores, indicating that besides recruitment of natural enemies for protection, plant phenotypic changes involved in trait-mediated interactions can entail costs for the plant in further interactions with other species in the community (Valladares *et al.* 2007; Utsumi & Ohgushi 2008; Frago & Godfray 2014). Therefore, plant trait-mediated indirect interaction networks may be viewed as an ultimate trade-off between intervening species (Schmitz *et al.* 2004).

Throughout the history of studies on plant-mediated multitrophic interactions, we have underestimated or ignored the importance of HAOs. The extended phenotype of HAOs that influences herbivores directly or plants indirectly may profoundly impact ecological processes. Our current study shows that parasitoid larvae, via their herbivore hosts, indirectly alter both plant transcriptional and metabolic responses to herbivory as well as interactions with hyperparasitoids. The effects of parasitoids living in the herbivores on plant responses to herbivory even override the effects of herbivore identity in HIPVs emission, which helps hyperparasitoids to locate their host. Future studies are required to elucidate the physiological changes in herbivores that result from parasitoid feeding within their bodies and to identify the key factors in herbivores that alter the plant responses to parasitized herbivores. Such studies are visibly important because our data show that parasitoid-related changes in plant phenotype have consequences for hyperparasitoids at the fourth trophic level and thus for biodiversity (Poelman *et al.* 2008a) and likely for community dynamics (cf. Van Zandt & Agrawal 2004; Poelman *et al.* 2010).

Acknowledgements

We thank Léon Westerd, Frans van Aggelen, and André Gidding for rearing the insects. This study was supported by the Earth and Life Sciences Council of the Netherlands Organisation for Scientific Research (NWO-ALW) by an Ecogenomics Grant 844.10.005 (to MD)

Supporting Information

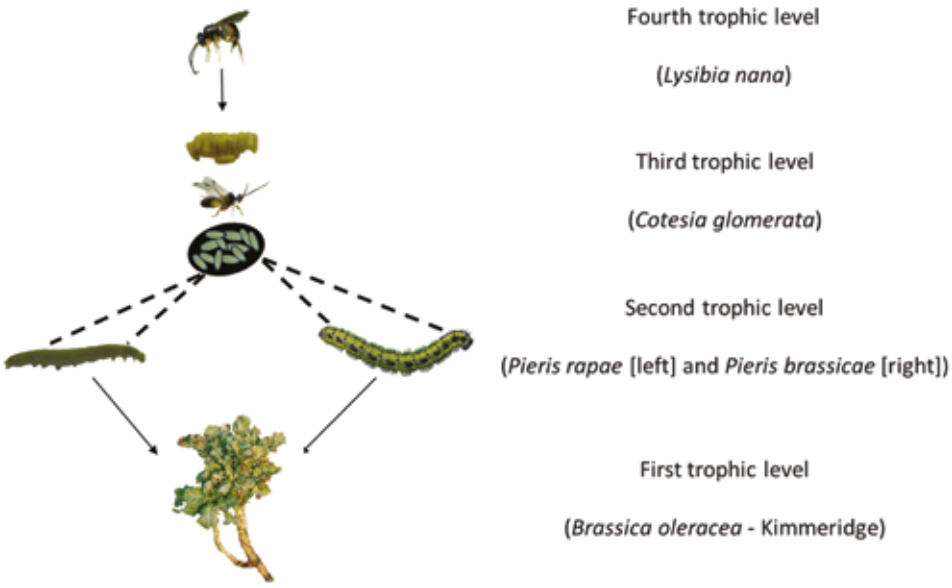

Figure S1: The four-trophic-level system used in this study.

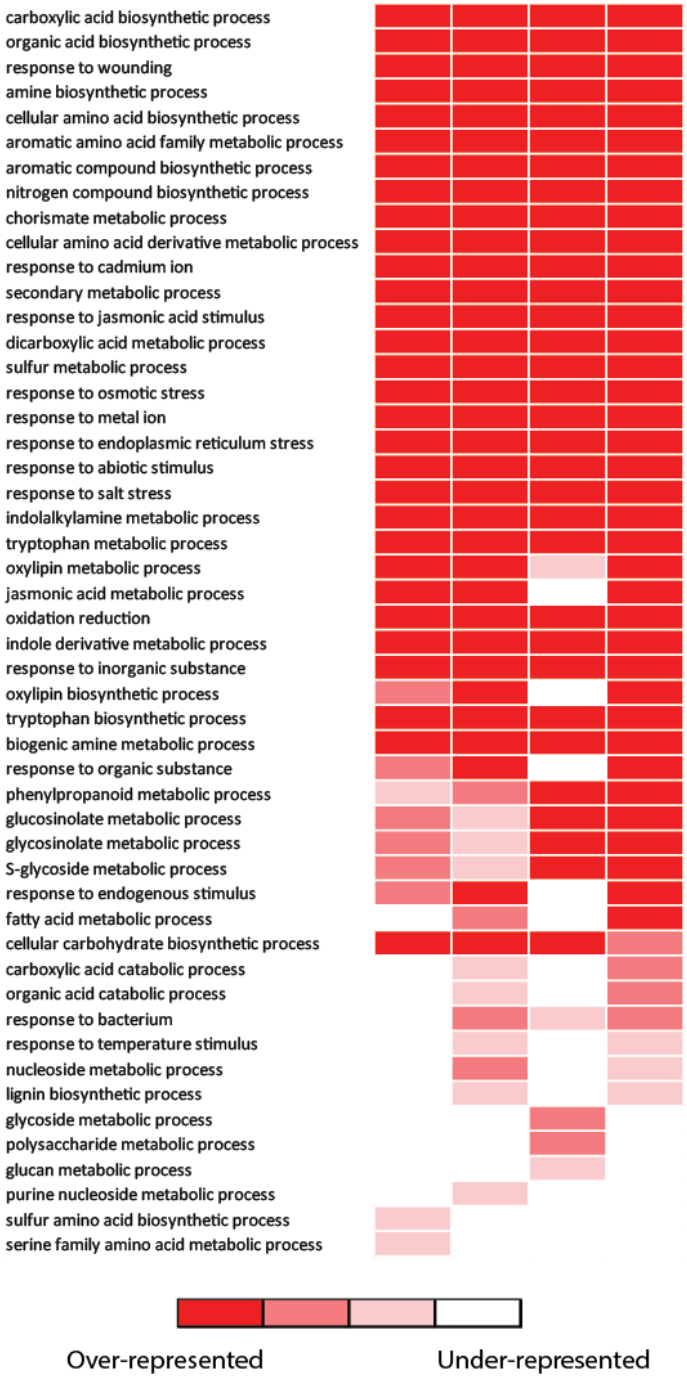

Figure S2: GO-enrichment analysis for biological processes using homologues (of *Arabidopsis thaliana*) of up-regulated *Brassica* unigenes in response to feeding damage by either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *Cotesia glomerata*-parasitized *P. rapae* (PR-CG), or *C. glomerata*-parasitized *P. brassicae* (PB-CG).

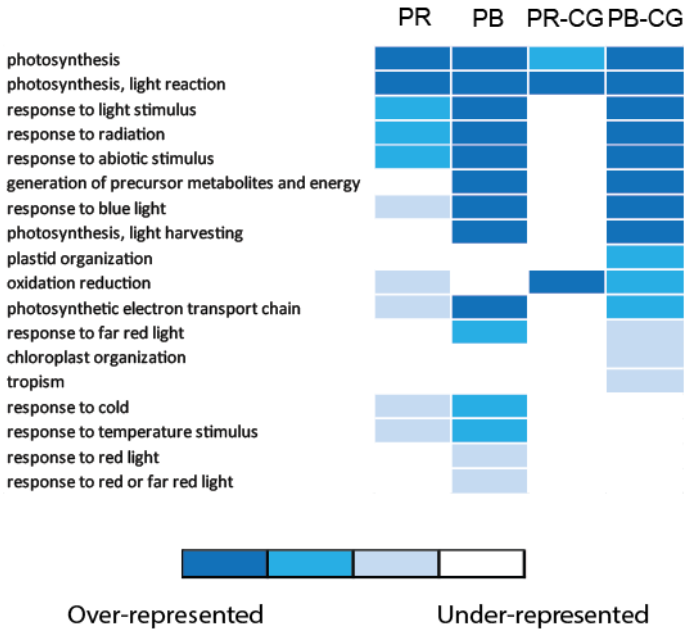

Figure S3: GO-enrichment analysis using homologues (of *Arabidopsis thaliana*) of down-regulated *Brassica* unigenes in response to feeding damage by either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *Cotesia glomerata*-parasitized *P. rapae* (PR-CG), or *C. glomerata*-parasitized *P. brassicae* (PB-CG).

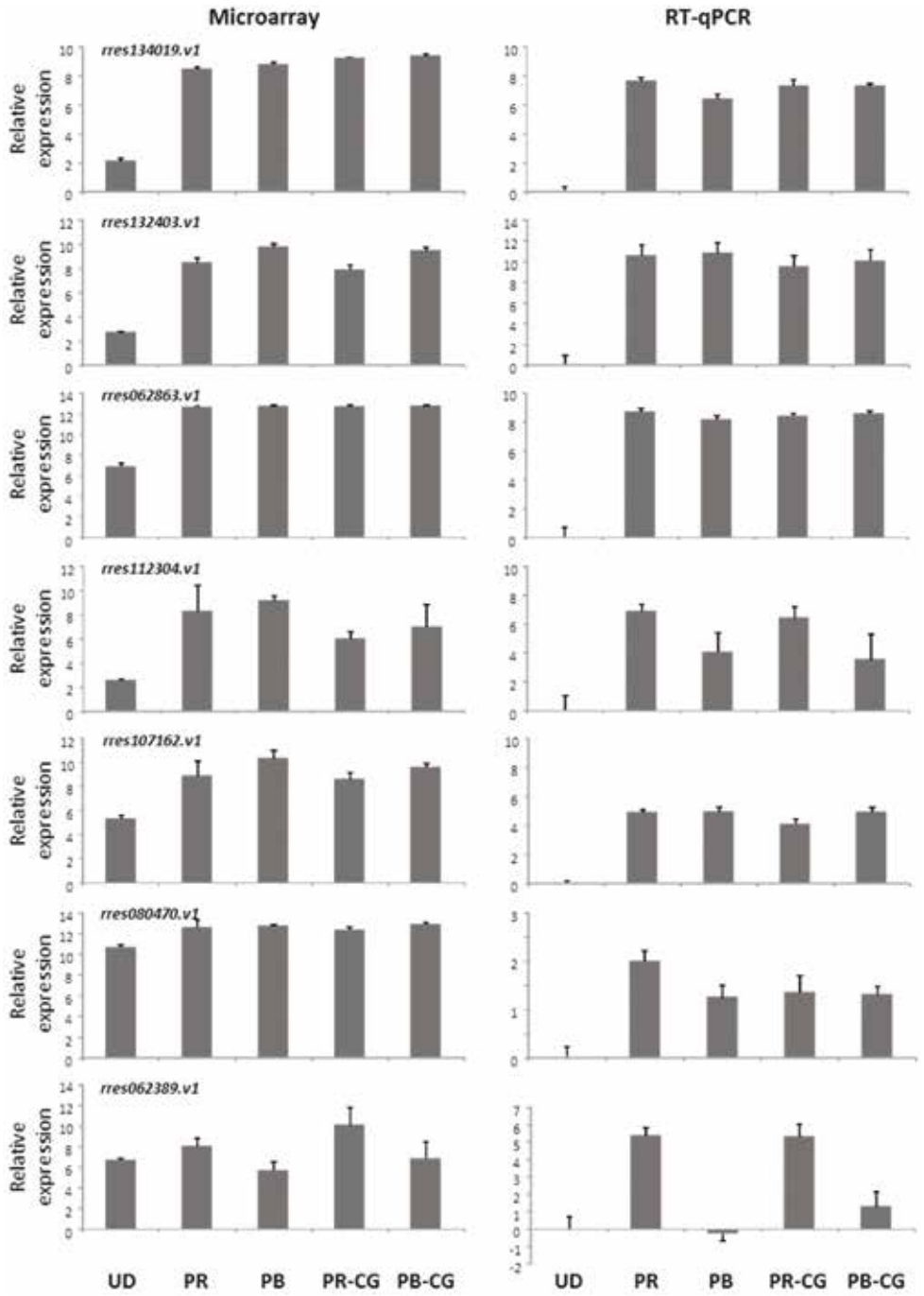

Figure S4: Validation of microarray gene expression data by quantitative real-time PCR. Plant materials were collected under the same conditions used for the microarray analysis after induction by either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *Cotesia glomerata*-parasitized *P. rapae* (PR-CG), or *C. glomerata*-parasitized *P. brassicae* caterpillars (PB-CG), or plants that had remained undamaged (UD). Relative expression is shown as log2 values.

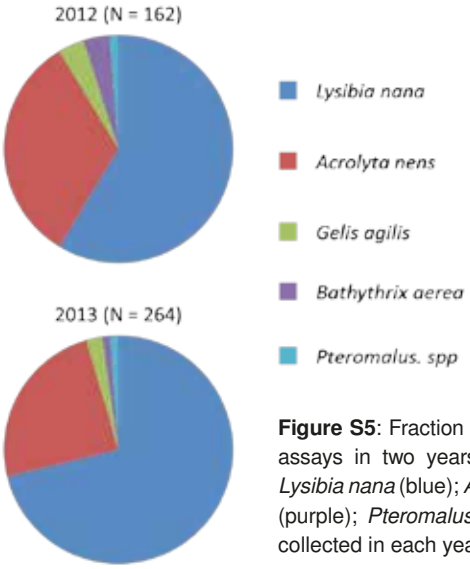

Figure S5: Fraction of each species of hyperparasitoid collected from field assays in two years. Colours indicate different hyperparasitoid species: *Lysibia nana* (blue); *Acrolyta nens* (red); *Gelis agilis* (green); *Bathythrix aerea* (purple); *Pteromalus spp.* (light blue). N: total number of hyperparasitoid collected in each year.

Table S1. List of primers used in microarray validation through qRT-PCR.

Primer name	Sequence (5' to 3')
rres134019F	GGGTGGAGCTTGCTTACCAG
rres134019R	ATGGTTCGTTGTGTTGGTGC
rres132403F	CTGGTATGGCCTTTGGTAACAC
rres132403R	CCAGCCTGAGAGGGTACTTC
rres062863F	GTTATCGATGCCGGGAGTTC
rres062863R	CCGGATGATCAGCATACGAA
rres112304F	GCAAGAGCAGGTCAAATCCC
rres112304R	ACCGATCTCTCCAAGTCCCA
rres107162F	ATACGCCAACGACGGTCTCT
rres107162R	AGGCTTGGAGCTCTTTGTCG
rres080470F	GATTTCCCATTTGCGAACGAC
rres080470R	ACTTGACCTGTTCCGCGTCT
rres062389F	AGCATTTGGGTCAAGCGTCT
rres062389R	TTGTTGACGAAATCGTTGCC

Table S2. Genes differentially expressed in *Cotesia glomerata*-parasitized *Pieris rapae* compared to unparasitized *P. rapae* treated plants. Relative changes in gene expression in leaves 24h after infestation by either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *C. glomerata*-parasitized *P. rapae* (PR-CG), *C. glomerata*-parasitized *P. brassicae* (PB-CG), or remained intact as undamaged control (UD). Mean expression ratios are calculated from three biologically independent replicates. AGI, Arabidopsis Genome Initiative. NA, not available.

Gene identification	*Brassica* unigene ID	AGI code	UD	PR	PB	PR-CG	PB-CG	Adjusted P-value (PR vs PR-CG)
Genes with a weaker expression in PR-CG compared to PR treated plants								
Transport								
Nodulin MtN21-like transporter family protein	rres052024.v1	AT4G01450	8.72	6.46	6.04	4.83	5.90	0.0398
NPR3	rres036334.v1	AT5G45110	8.02	7.93	7.46	6.13	7.34	0.0437
Other process								
Plant invertase/pectin methylesterase inhibitor superfamily	rres044140.v1	AT1G23200	7.40	7.60	7.64	6.29	7.15	0.0369
proline-rich family protein	rres084690.v1	AT5G45350	9.06	8.89	9.04	7.98	8.70	0.0366
member of CYP81H	rres128636.v1	AT4G37310	7.82	6.42	6.66	5.52	6.09	0.0437
ARM repeat superfamily protein	rres082415.v1	AT3G03440	2.44	2.93	2.79	2.15	2.46	0.0261
Unknown	rres094673.v1	AT4G15990	6.49	6.70	6.85	5.80	6.44	0.0221
Unknown	rres047765.v1	AT3G51510	9.50	9.39	9.00	8.61	8.66	0.0437
Unknown	rres142560.v1	AT5G55620	11.28	9.95	9.58	9.00	9.11	0.0366
Unknown	rres110927.v1	AT1G64680	10.18	10.38	10.30	9.68	9.90	0.0366
NA	rres010149.v1	NA	10.42	10.02	9.42	8.88	9.49	0.0176
NA	rres024098.v1	NA	7.76	6.76	6.76	5.62	6.25	0.0437
NA	rres020825.v1	NA	6.54	7.51	7.21	6.40	6.62	0.0437
NA	rres011961.v1	NA	10.84	10.49	9.94	9.48	9.83	0.0221
NA	rres040567.v1	NA	4.19	4.22	4.68	3.42	4.16	0.0396
Genes with a stronger expression in PR-CG compared to PR treated plants								
Transcription factor								
a basic helix-loop-helix (bHLH) transcription factor	rres062109.v1	AT5G53210	1.73	1.83	1.76	2.68	1.88	0.0261
Sequence-specific DNA binding transcription factor activity	rres136822.v1	AT5G46880	3.57	3.78	3.24	4.89	3.91	0.0369
a member of the BZIP family of transcription factors	rres036391.v1	AT3G58120	8.70	6.91	6.26	8.65	7.07	0.0467
Protein metabolism/ metabolism								
ClpX3, a subunit of the Clp protease complex	rres103403.v1	AT1G33360	5.66	5.65	5.80	6.49	6.16	0.0437

a member of the 3-ketoacyl-CoA synthase family involved in the iosynthesis of VLCFA	rres095354.v1	AT1G04220	4.85	4.94	4.69	6.34	5.14	0.0176
predicted to encode subunit 6 of mitochondrial complex II	rres035970.v1	AT1G08480	5.18	5.19	5.69	6.50	6.23	0.0437
embryo defective 2296	rres057056.v1	AT2G18020	7.51	7.87	8.23	8.49	8.39	0.0437
endo-1,4-beta-glucanase	rres100896.v1	AT1G70710	6.89	7.52	7.70	8.35	7.84	0.0319
Leucine-rich repeat protein kinase family protein	rres066810.v1	AT2G41820	4.67	3.79	3.81	4.89	4.06	0.0366
Ribosomal protein S24e family protein	rres121006.v1	AT3G04920	7.64	8.24	8.58	9.11	8.89	0.0441
Li-tolerant lipase 1	rres099404.v1	AT3G04290	2.78	2.97	2.46	3.95	2.87	0.0437
SGNH hydrolase-type esterase superfamily protein	rres118102.v1	AT2G40250	2.16	1.93	2.02	2.83	2.36	0.0396
TPX2 (targeting protein for Xklp2) protein family	rres099118.v1	AT2G35880	8.45	8.68	8.80	9.32	9.03	0.0437
KCBP-interacting protein kinase interacts specifically with the tail region of KCBP	rres052291.v1	AT3G52890	5.07	3.82	5.06	5.76	5.09	0.0017
early-responsive to dehydration stress family protein	rres048675.v1	AT4G15430	4.38	4.06	4.55	5.26	4.14	0.0245
Germin-like protein 3	rres101760.v1	AT5G20630	7.33	5.13	5.53	6.94	6.54	0.0437
mitochondrial phosphatidylserine decarboxylase	rres118787.v1	AT4G16700	2.76	2.58	3.15	3.39	3.02	0.0368
LysM-containing receptor-like kinase	rres046671.v1	AT1G51940	5.13	4.61	4.96	5.51	5.21	0.0437
sn-glycerol-3-phosphate 2-O-acyltransferase	rres100736.v1	AT2G38110	3.42	3.23	3.53	4.36	3.69	0.0368
nodulin MtN21-like transporter family protein	rres007807.v1	AT3G28080	6.44	5.45	5.55	7.08	6.28	0.0437
Chloroplast RNA-binding protein 29	rres049487.v1	AT3G53460	8.43	7.32	7.52	8.25	7.89	0.0437
N-terminal protein myristoylation	rres113066.v1	AT4G13540	4.90	4.12	4.15	5.37	4.58	0.0221
Aldolase-type TIM barrel family protein	rres071906.v1	AT5G48220	5.94	5.90	6.57	7.54	7.16	0.0123
Calcium-binding EF-hand family protein	rres051097.v1	AT4G26470	2.74	2.88	3.03	3.70	3.05	0.0494
endo-1,4-beta-glucanase	rres134063.v1	AT1G70710	4.79	5.63	5.33	6.72	5.69	0.0437
Galactosyltransferase family protein	rres063397.v1	AT5G62620	2.39	2.75	2.81	3.70	2.78	0.0440
Transport								
Nuclear transport factor 2 (NTF2) family protein with RNA binding (RRM-RBD-RNP motifs) domain	rres086417.v1	AT1G13730	4.33	4.70	4.50	5.75	4.72	0.0400
GDSL-like Lipase/Acylhydrolase superfamily protein	rres107453.v1	AT1G29660	11.45	10.93	10.84	11.70	11.12	0.0437
Homologous to receptor protein kinases	rres032407.v1	AT2G26330	2.35	2.50	2.31	3.55	2.45	0.0319
Mog1/PsbP/DUF1795-like photosystem II reaction center PsbP family protein	rres020036.v1	AT5G11450	9.39	8.86	8.96	9.66	9.38	0.0437
chalcone synthase	rres034258.v1	AT5G13930	5.39	5.60	5.26	7.06	6.19	0.0437

Other process								
Tetratricopeptide repeat (TPR)-like superfamily protein	rres062017.v1	AT1G31790	3.90	3.83	4.08	4.56	4.25	0.0437
Nucleotide-diphospho-sugar transferases superfamily protein	rres032703.v1	AT2G19880	2.24	2.37	2.59	3.21	2.65	0.0366
RNA-binding (RRM/RBD/RNP motifs) family protein	rres077871.v1	AT1G05970	5.62	5.15	5.86	6.06	5.92	0.0494
RNA-binding (RRM/RBD/RNP motifs) family protein	rres063387.v1	AT4G35785	7.08	6.95	7.10	7.91	7.60	0.0437
Arabidopsis expansin 5	rres071905.v1	AT3G29030	6.65	6.78	6.50	7.79	6.64	0.0500
a member of the TBL (TRICHOME BIREFRINGENCE-LIKE) gene family	rres128328.v1	AT2G42570	4.78	5.00	4.90	6.05	5.14	0.0194
Leucine-rich repeat (LRR) family protein	rres113537.v1	AT5G23400	5.44	5.28	5.30	6.33	5.35	0.0366
nucleosome assembly protein	rres101215.v1	AT2G19480	4.84	4.54	5.09	5.61	5.45	0.0194
Plasma-membrane choline transporter family protein	rres036431.v1	AT3G15380	2.40	2.21	2.41	3.32	2.77	0.0176
a component of the putative Arabidopsis THO/TREX complex	rres031745.v1	AT1G24706	3.21	2.81	3.48	3.99	3.48	0.0437
Myosin heavy chain-related protein	rres032306.v1	AT5G41140	4.88	4.36	4.74	5.59	5.40	0.0437
Pectin lyase-like superfamily protein	rres041802.v1	AT5G63180	4.34	3.96	4.43	5.34	4.74	0.0194
alpha/beta-Hydrolases superfamily protein	rres134748.v1	AT1G72620	6.02	3.98	3.80	5.38	4.05	0.0437
unknown	rres084276.v1	AT4G39900	8.39	7.59	7.12	8.54	7.65	0.0437
unknown	rres100690.v1	AT5G47920	3.29	3.08	3.60	4.54	4.13	0.0221
unknown	rres064987.v1	AT4G21215	5.56	5.13	5.19	6.14	5.24	0.0369
unknown	rres064479.v1	AT3G52110	2.47	2.56	2.69	3.41	2.75	0.0437
unknown	rres019962.v1	AT5G21920	7.18	6.50	7.02	7.99	7.86	0.0176
unknown	rres089454.v1	AT5G62170	8.41	7.22	6.84	7.92	7.01	0.0437
NA	rres007363.v1	NA	2.39	2.49	2.44	3.99	2.73	0.0194
NA	rres133661.v1	NA	3.31	2.96	3.53	4.30	3.96	0.0437
NA	rres013651.v1	NA	7.69	7.28	7.00	8.53	7.90	0.0199
NA	rres079268.v1	NA	6.15	5.53	5.70	6.67	6.19	0.0486
NA	rres077144.v1	NA	2.75	2.04	2.02	3.15	2.41	0.0366
NA	rres030085.v1	NA	3.68	3.63	3.62	4.67	4.00	0.0437
NA	rres015288.v1	NA	6.89	7.07	7.15	7.67	7.41	0.0437
NA	rres024953.v1	NA	2.39	1.68	2.23	2.37	2.55	0.0437

Table S3. Genes differentially expressed in *Cotesia glomerata*-parasitized *Pieris brassicae* (PB-CG) compared to *C. glomerata* parasitized *P. rapae* (PR-CG) treated plants. Relative changes in gene expression in leaves 24h after infestation by either unparasitized *P. rapae* (PR), unparasitized *P. brassicae* (PB), *C. glomerata*-parasitized *P. rapae* (PR-CG), *C. glomerata*-parasitized *P. brassicae* (PB-CG), or remained intact as undamaged control (UD). Mean expression ratios are calculated from three biologically independent replicates. AGI, Arabidopsis Genome Initiative. NA, not available.

Gene identification	*Brassica* unigene ID	AGI code	UD	PR	PB	PR-CG	PB-CG	Adjusted P-value (PB-CG vs PR-CG)
Genes with a weaker expression in PB-CG compared to PR-CG treated plants								
Transcription factor								
Helix-loop-helix (bHLH) transcription factor	rres062109.v1	AT5G53210	1.73	1.83	1.76	2.68	1.88	0.0419
Transport								
Homologous to receptor protein kinases	rres032407.v1	AT2G26330	2.35	2.50	2.31	3.55	2.45	0.0371
Manganese tracking factor for mitochondrial SOD2 (MTM1)	rres095989.v1	AT4G27940	9.54	9.38	8.82	9.07	8.20	0.0419
Protein metabolism/ metabolism								
Asparaginase B1	rres122797.v1	AT3G16150	3.57	3.05	2.63	3.71	2.82	0.0419
3-ketoacyl-CoA synthase 2	rres095354.v1	AT1G04220	4.85	4.94	4.69	6.34	5.14	0.0417
Li-tolerant lipase 1 (LTL1)	rres099404.v1	AT3G04290	2.78	2.97	2.46	3.95	2.87	0.0419
Plant invertase/pectin methylesterase inhibitor superfamily	rres102271.v1	AT3G49220	8.54	8.28	7.67	8.38	7.51	0.0443
ERD (early-responsive to dehydration stress) family protein	rres048675.v1	AT4G15430	4.38	4.06	4.55	5.26	4.14	0.0419
ERD (early-responsive to dehydration stress) family protein	rres055956.v1	AT4G15430	5.01	4.75	4.32	5.61	4.22	0.0382
Other process								
Arabidopsis thaliana expansin 5	rres071905.v1	AT3G29030	6.65	6.78	6.50	7.79	6.64	0.0419
Member of the TBL (TRICHOME BIREFRINGENCE-LIKE) gene family	rres128328.v1	AT2G42570	4.78	5.00	4.90	6.05	5.14	0.0419
Pectate lyase family protein	rres070095.v1	AT1G67750	7.30	6.70	6.14	7.04	6.19	0.0419
Leucine-rich repeat (LRR) family protein	rres032832.v1	AT4G18670	5.60	6.39	6.11	6.96	6.28	0.0419
Unknown	rres089454.v1	AT5G62170	8.41	7.22	6.84	7.92	7.01	0.0239
Unknown	rres068060.v1	AT5G59020	4.40	5.35	5.38	5.93	5.03	0.0419
NA	rres014113.v1	NA	6.92	6.18	5.81	7.21	5.74	0.0371
NA	rres007363.v1	NA	2.39	2.49	2.44	3.99	2.73	0.0463
NA	rres086579.v1	NA	7.43	6.87	6.38	7.23	6.26	0.0239
NA	rres015856.v1	NA	9.20	8.42	8.22	9.04	8.18	0.0239

Genes with a stronger expression in PB-CG compared to PR-CG treated plants								
Transcription factor								
NAC transcription factor induced by drought, high salt, and abscisic acid	rres115216.v1	AT1G52890	6.95	8.43	8.90	7.51	8.69	0.0419
Transport								
Putative blue light receptor protein	rres093849.v1	AT2G02710	7.45	7.66	8.26	6.74	7.85	0.0443
Potassium transporter 1	rres095212.v1	AT2G26650	7.44	10.11	10.68	9.68	10.53	0.0419
Protein metabolism/ metabolism								
Glutamate decarboxylase 1	rres060461.v1	AT5G17330	4.86	5.40	6.34	4.79	7.57	0.0417
Jasmonate-zim-domain protein 12 (JAZ12)	rres091939.v1	AT5G20900	5.20	5.61	5.14	3.37	6.60	0.0419
Beta glucosidase 29 (BGLU29);	rres085843.v1	AT2G44470	3.42	6.40	6.99	5.95	7.43	0.0239
Polyamine oxidase 1	rres127260.v1	AT5G13700	5.85	8.10	9.47	8.00	9.34	0.0419
Polyamine oxidase 1	rres140778.v1	AT5G13700	3.96	5.48	6.54	4.83	6.46	0.0261
Member of CYP81D family of cytochrome p450s	rres078442.v1	AT5G36220	6.39	9.64	9.99	9.17	10.08	0.0419
Other process								
RARE-COLD-INDUCIBLE 2B (RCI2B)	rres109929.v1	AT3G05890	2.71	5.68	6.81	5.45	6.62	0.0419
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	rres038199.v1	AT5G04380	2.77	4.46	5.16	3.75	5.64	0.0239
Unknown	rres092185.v1	AT1G61240	5.62	7.05	7.80	6.85	8.04	0.0239
Unknown	rres072503.v1	AT2G42760	4.12	6.37	7.21	6.14	7.13	0.0239

Table S4. Genes differentially expressed, due to herbivre species, in unparasitized or *Cotesia glomerata*-parasitized *Pieris brassicae* compared to unparasitized or *C. glomerata*-parasitized *P. rapae* treated plants. Relative changes in gene expression in leaves 24h after infestation by either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *C. glomerata*-parasitized *P. rapae* (PR-CG), *C. glomerata*-parasitized *P. brassicae* (PB-CG), or remained intact as undamaged control (UD). Mean expression ratios are calculated from three biologically independent replicates. AGI, Arabidopsis Genome Initiative. Adjusted P-value indicates the effect of herbivore identity on expression of particular plant gene. NA, not available.

Gene identification	*Brassica* unigene ID	AGI code	UD	PR	PB	PR-CG	PB-CG	Adjusted P-value
Genes with a weaker expression in PB or PB-CG compared to PR or PR-CG treated plants								
Transcription factor								
ICE2 (Inducer of CBF Expression 2), a transcription factor of the bHLH family	rres066150.v1	AT1G12860	9.35	7.96	1.76	8.02	7.28	0.0420
Required for control of cell proliferation and encodes a putative transcriptional regulator similar to AP2	rres057945.v1	AT4G37750	5.63	5.57	4.97	6.04	4.98	0.0497
Ca(2+)-dependent CaM-binding protein	rres086642.v1	AT5G28300	6.60	6.99	6.29	7.40	6.61	0.0420
Golden2-like 2, regulate the expression of the photosynthetic apparatus	rres111113.v1	AT5G44190	10.51	9.12	8.57	9.11	8.09	0.0420
Protein metabolism/ metabolism								
PQ-loop repeat family protein	rres063537.v1	AT2G41050	7.44	6.80	6.16	6.52	5.81	0.0497
Actin-11, expressed predominantly during reproductive development	rres073139.v1	AT3G12110	11.21	10.67	10.26	10.78	10.18	0.0273
RNA tetratricopeptide repeat-containing protein	rres130141.v1	AT3G17040	6.25	6.12	5.51	5.97	5.45	0.0420
Eukaryotic aspartyl protease family protein	rres055992.v1	AT3G54400	7.41	6.87	6.02	7.00	5.91	0.0420
3-ketoacyl-CoA synthase 2	rres073591.v1	AT1G04220	9.30	9.39	8.83	9.83	9.07	0.0459
Mitochondrial branched-chain amino acid aminotransferase	rres108903.v1	AT1G10060	8.64	8.70	7.95	9.12	8.27	0.0420
Mitochondrial branched-chain amino acid aminotransferase	rres107710.v1	AT1G10060	7.83	7.79	7.12	8.19	7.34	0.0497
Chlorophyllide a oxygenase	rres091183.v1	AT1G44446	9.31	8.76	8.18	8.64	8.11	0.0320
Protein with glutamyl-tRNA reductase (GluTR) activity	rres088473.v1	AT1G58290	7.40	7.29	6.74	7.32	6.70	0.0205
Protein with glutamyl-tRNA reductase (GluTR) activity	rres089660.v1	AT1G58290	8.45	8.20	7.69	8.23	7.62	0.0297
Chloroplastic copper/zinc superoxide dismutase	rres108012.v1	AT2G28190	7.77	7.86	7.10	8.20	7.23	0.0309
Protein predicted to function in tandem with PDX2 to form glutamine amidotransferase complex	rres079357.v1	AT2G38230	8.61	7.04	6.50	7.05	6.33	0.0420
Acyl carrier protein	rres085584.v1	AT3G05020	5.12	5.24	4.71	5.43	4.87	0.0497

Chloroplastic enzyme responsible for the synthesis of 16:3 and 18:3 fatty acids from galactolipids	rres075487.v1	AT3G11170	5.20	4.68	4.18	4.90	4.08	0.0420
Pectin lyase-like superfamily protein	rres120525.v1	AT4G23820	6.12	4.97	4.31	5.53	4.42	0.0497
A component of the fatty acid elongation machinery required for C28 to C30 fatty acid elongation	rres035590.v1	AT4G24510	8.25	8.03	6.99	8.20	7.44	0.0194
GASA domain containing protein	rres070947.v1	AT5G14920	6.83	6.62	5.93	7.05	5.72	0.0497
GDSL-like Lipase/Acylhydrolase superfamily protein	rres075139.v1	AT5G33370	6.16	7.78	5.32	9.40	6.30	0.0497
9-CIS EPOXYCAROTENOID DIOXYGENASE DEFECTIVE 1	rres050663.v1	AT1G64670	7.63	7.63	7.03	8.06	7.13	0.0420
Plant invertase/pectin methylesterase inhibitor superfamily	rres102271.v1	AT3G49220	8.54	8.28	7.67	8.38	7.51	0.0100
Plant invertase/pectin methylesterase inhibitor superfamily	rres069007.v1	AT3G49220	9.73	9.52	8.94	9.49	8.80	0.0320
Plant invertase/pectin methylesterase inhibitor superfamily	rres097616.v1	AT3G49220	9.47	9.30	8.76	9.15	8.50	0.0420
GUN, genomes uncoupled	rres127281.v1	AT3G59400	7.69	6.96	6.50	7.05	6.32	0.0374
Transport								
Plasma membrane intrinsic protein subfamily PIP1	rres037528.v1	AT1G01620	8.74	7.89	7.47	8.13	7.18	0.0420
Phytochrome kinase substrate 2	rres083880.v1	AT1G14280	8.19	6.92	6.02	7.06	6.26	0.0297
Phytochrome kinase substrate 2	rres062304.v1	AT1G14280	4.06	2.88	2.26	3.12	2.35	0.0262
Lhcb6 protein (Lhcb6), light harvesting complex of photosystem II	rres082214.v1	AT1G15820	8.16	7.39	7.06	7.70	6.89	0.0497
Arabidopsis thaliana expansin 6	rres102441.v1	AT2G28950	9.58	9.46	8.88	9.40	8.91	0.0351
ACT2, involved in tip growth of root hairs	rres101594.v1	AT3G18780	10.39	9.78	9.28	9.85	9.29	0.0497
Cell wall-plasma membrane linker protein homolog (CWLP)	rres063985.v1	AT3G22120	9.98	9.60	9.13	9.79	8.96	0.0497
light-harvesting chlorophyll a/b-binding (LHC) protein	rres139092.v1	AT3G27690	11.37	10.57	10.08	10.61	10.13	0.0262
plasma membrane intrinsic protein subfamily PIP1	rres134242.v1	AT3G61430	10.33	9.74	9.19	9.80	9.25	0.0262
Light-harvesting chlorophyll a/b binding protein CP26	rres087037.v1	AT4G10340	6.79	5.63	4.44	5.60	4.58	0.0117
Methyltransferase, responsible for the methylation of magnesium protoporphyrin IX	rres040862.v1	AT4G25080	8.39	7.40	6.93	7.34	6.78	0.0420
P-glycoprotein 2 (PGP2);	rres117545.v1	AT4G25960	8.40	7.82	7.17	8.04	7.29	0.0439
Manganese tracking factor for mitochondrial SOD2 (MTM1)	rres041686.v1	AT4G27940	8.24	8.21	7.44	7.82	6.95	0.0452
Manganese tracking factor for mitochondrial SOD2 (MTM1)	rres034040.v1	AT4G27940	8.08	8.13	7.57	7.91	7.17	0.0420
Plasma membrane intrinsic protein PIP 3	rres042428.v1	AT4G35100	8.56	7.38	6.92	7.51	6.86	0.0205
Heavy metal transport/detoxification superfamily protein	rres071301.v1	AT5G19090	5.33	5.52	4.91	5.48	5.13	0.0488
SLAH3 protein, involved in ion homeostasis in guard cells	rres039725.v1	AT5G24030	9.09	8.15	7.42	8.38	7.38	0.0283
Transcription regulator responsible for specific upregulation of the translocon genes	rres066342.v1	AT5G57180	9.08	7.89	7.48	8.06	7.32	0.0409

Major facilitator superfamily protein	rres076150.v1	AT5G62730	10.08	7.30	6.66	7.64	6.62	0.0283
Other process								
Thaumatin-like protein	rres079846.v1	AT1G18250	5.68	5.81	5.19	6.11	5.36	0.0262
X8-GPI family of proteins	rres049567.v1	AT1G18650	9.86	8.52	7.75	8.62	8.05	0.0420
Atypical arabinogalactan protein	rres103286.v1	AT1G28290	6.56	6.84	6.14	6.78	6.28	0.0478
Pectate lyase family protein	rres070095.v1	AT1G67750	7.30	6.70	6.14	7.04	6.19	0.0205
Cysteine rich protein having antimicrobial properties	rres042310.v1	AT1G72260	3.86	4.33	3.67	4.27	3.75	0.0400
Actin-like ATPase superfamily protein	rres105424.v1	AT2G42100	9.37	8.74	8.22	8.77	8.12	0.0403
RNA-binding (RRM/RBD/RNP motifs) family protein	rres139997.v1	AT2G46780	5.37	4.98	4.20	5.28	4.48	0.0262
Glutaredoxin family protein	rres108269.v1	AT2G47880	4.96	5.12	4.46	5.41	4.63	0.0262
Protein localized to phloem filaments, required for phloem filament formation	rres075855.v1	AT3G01670	7.44	7.54	7.14	7.82	7.21	0.0493
P-loop containing nucleoside triphosphate hydrolases superfamily protein	rres106133.v1	AT3G45850	5.27	5.67	4.96	5.88	4.98	0.0206
selenium-binding protein 2 (SBP2)	rres066393.v1	AT4G14040	6.00	5.86	5.45	5.79	5.16	0.0420
Leucine-rich repeat (LRR) family protein	rres074246.v1	AT4G18670	6.41	7.21	6.75	7.14	6.60	0.0452
Expansin -like protein	rres048764.v1	AT4G38210	4.84	5.14	4.60	5.20	4.59	0.0493
SAUR-like auxin-responsive protein family	rres128864.v1	AT5G18030	5.83	5.58	4.92	5.70	4.98	0.0420
PR (pathogenesis-related) protein	rres130840.v1	AT5G36910	6.96	7.32	6.47	7.26	6.72	0.0262
Unknown	rres050262.v1	AT1G12845	5.50	5.66	5.18	5.85	5.10	0.0420
Unknown	rres032160.v1	AT2G20820	4.27	5.15	4.49	4.97	4.66	0.0497
Unknown	rres134479.v1	AT1G56580	7.15	7.78	7.38	7.86	7.46	0.0420
Unknown	rres048536.v1	AT4G29310	5.45	5.29	4.59	5.59	4.80	0.0400
Unknown	rres098658.v1	AT5G13720	10.39	9.75	9.33	9.73	9.31	0.0294
NA	rres023699.v1	NA	4.54	4.83	3.60	4.57	3.68	0.0497
NA	rres017697.v1	NA	5.01	6.09	5.18	5.95	4.85	0.0328
NA	rres009092.v1	NA	4.64	5.08	3.93	4.53	3.85	0.0497
NA	rres086579.v1	NA	7.43	6.87	6.38	7.23	6.26	0.0166
NA	rres018197.v1	NA	6.19	6.02	5.38	6.10	5.47	0.0485
NA	rres093554.v1	NA	4.71	4.94	4.47	5.23	4.50	0.0420
NA	rres125784.v1	NA	5.26	4.83	4.34	4.91	4.22	0.0497
NA	rres015603.v1	NA	8.39	7.28	6.55	7.15	6.71	0.0420
NA	rres116808.v1	NA	5.01	5.22	4.65	5.10	4.54	0.0420

Genes with a stronger expression in PB or PB-CG compared to PR or PR-CG treated plants								
Transcription factor								
ATMYC2, MYC-related transcriptional activator	rres082589.v1	AT1G32640	6.01	8.03	8.78	7.78	8.52	0.0262
ATMYC2, MYC-related transcriptional activator	rres067309.v1	AT1G32640	5.75	7.92	8.73	7.80	8.52	0.0205
MADS domain protein	rres141865.v1	AT1G77080	2.81	3.08	4.35	2.24	4.10	0.0420
Scarecrow-like protein (SCL13)	rres110881.v1	AT4G17230	5.82	6.09	6.61	6.16	6.56	0.0490
DREB subfamily A-6 of ERF/AP2 transcription factor family	rres071465.v1	AT4G28140	2.44	2.30	2.86	2.39	2.79	0.0262
WRKY Transcription Factor	rres037279.v1	AT4G31550	3.47	3.77	4.41	3.82	4.38	0.0420
ERF (ethylene response factor) subfamily B-4 of ERF/AP2 transcription factor family	rres066026.v1	AT5G13330	3.13	3.33	4.69	3.78	4.63	0.0439
TLP family	rres092037.v1	AT5G18680	6.89	7.82	8.24	7.59	8.41	0.0497
Protein metabolism/ metabolism								
IAA-amino acid conjugate hydrolase subfamily	rres127842.v1	AT1G51760	4.54	7.75	8.36	7.73	8.34	0.0262
P-loop containing nucleoside triphosphate hydrolases superfamily protein	rres045648.v1	AT2G03750	10.23	10.99	11.52	10.90	11.62	0.0331
AP2C1, belongs to the clade B of the PP2C-superfamily	rres119373.v1	AT2G30020	3.18	3.88	4.87	3.81	4.82	0.0117
Protein kinase superfamily protein	rres135461.v1	AT3G61960	8.96	9.86	10.27	9.89	10.52	0.0497
Protein kinase superfamily protein	rres093998.v1	AT3G61960	7.31	8.28	8.78	8.22	8.96	0.0296
S-adenosyl-L-homocysteine hydrolase required for DNA methylation-dependent gene silencing	rres091584.v1	AT4G13940	9.26	10.96	11.36	10.95	11.37	0.0420
RING/U-box superfamily protein	rres048488.v1	AT4G28890	4.86	5.58	6.52	5.49	6.44	0.0137
Receptor-like cytoplasmic kinase	rres045255.v1	AT4G35600	8.44	9.08	9.39	8.87	9.43	0.0400
MEKK subfamily	rres123853.v1	AT4G36950	3.54	4.88	6.09	5.11	5.86	0.0262
ATP-dependent Clp protease regulatory subunit	rres093775.v1	AT5G51070	8.15	8.94	9.56	8.84	9.45	0.0473
Calcium-dependent Protein Kinase	rres127493.v1	AT3G57530	6.75	6.77	7.64	6.99	7.38	0.0420
Seven-transmembrane domain proteins	rres086142.v1	AT1G11310	4.85	5.35	5.84	5.46	5.83	0.0420
Lactoylglutathione lyase / glyoxalase I family protein	rres064167.v1	AT1G15380	3.50	5.71	7.39	5.67	6.96	0.0329
F-box protein family	rres084078.v1	AT1G15670	3.52	3.47	4.44	3.45	4.02	0.0490
Jasmonate-zim-domain protein 5 (JAZ5)	rres142090.v1	AT1G17380	4.27	8.03	9.24	8.25	9.01	0.0166
Phosphoethanolamine/phosphocholine phosphatase	rres113688.v1	AT1G17710	4.30	4.87	5.74	4.66	5.30	0.0497
Jasmonate-zim-domain protein 1 (JAZ1)	rres139769.v1	AT1G19180	6.62	9.20	9.80	8.91	9.69	0.0420
Jasmonate-zim-domain protein 8 (JAZ8)	rres041544.v1	AT1G30135	1.78	2.65	3.43	2.55	3.08	0.0400

Midchain alkane hydroxylase	rres129700.v1	AT1G57750	4.36	7.10	7.70	7.06	7.57	0.0453
PLAT/LH2 domain-containing lipoxygenase family protein (ATLOX6)	rres092894.v1	AT1G67560	6.16	8.34	8.86	8.25	8.70	0.0420
PLAT/LH2 domain-containing lipoxygenase family protein (ATLOX4)	rres073806.v1	AT1G72520	6.28	8.96	10.06	8.60	9.61	0.0497
12-oxophytodienoate reductase	rres123222.v1	AT2G06050	7.63	9.59	10.12	9.69	10.12	0.0262
Tyrosine aminotransferase	rres124318.v1	AT2G24850	3.72	6.28	7.16	6.26	7.31	0.0359
NAD(P)-binding Rossmann-fold superfamily protein	rres074452.v1	AT2G30670	3.45	6.46	8.19	5.76	7.79	0.0117
Methionine adenosyltransferase 3 (MAT3)	rres063813.v1	AT2G36880	7.12	9.61	10.16	9.87	10.30	0.0400
Beta glucosidase 15 (BGLU15);	rres053053.v1	AT2G44450	2.98	7.50	8.12	7.46	7.91	0.0294
Beta glucosidase 29 (BGLU29)	rres085843.v1	AT2G44470	3.42	6.40	6.99	5.95	7.43	0.0277
Reticulon family protein	rres087811.v1	AT2G46170	7.10	7.91	8.40	7.87	8.49	0.0497
Lysophosphatidic acid phosphatase	rres100575.v1	T3G03520	4.96	7.77	8.64	7.51	8.34	0.0309
Lysophosphatidic acid phosphatase	rres087880.v1	AT3G03520	6.64	9.91	10.82	9.92	10.75	0.0021
9-cis-epoxycarotenoid dioxygenase	rres040123.v1	AT3G14440	5.90	5.93	6.74	5.54	6.56	0.0294
Jasmonate-zim-domain protein 3 (JAZ3)	rres121963.v1	AT3G17860	7.54	8.71	9.22	8.53	9.12	0.0275
Raffinose-specific alpha-galactosidase	rres059537.v1	AT3G57520	5.26	8.33	9.22	7.92	9.08	0.0297
NAD-dependent malic enzyme (NAD-ME)	rres125925.v1	AT4G00570	7.39	8.54	8.92	8.47	8.85	0.0431
Geranylgeranyl reductase (GGR)	rres048152.v1	AT4G38460	5.36	4.94	5.54	4.98	5.50	0.0166
GDSL-like Lipase/Acylhydrolase superfamily protein	rres140504.v1	AT5G03610	1.99	4.01	6.73	4.84	5.81	0.0420
Jasmonate-zim-domain protein 10 (JAZ10)	rres055159.v1	AT5G13220	4.66	8.20	10.12	7.71	9.68	0.0262
protein with polyamine oxidase activity	rres035972.v1	AT5G13700	3.59	3.69	4.87	3.78	4.80	0.0262
protein with polyamine oxidase activity	rres140778.v1	AT5G13700	3.96	5.48	6.54	4.83	6.46	0.0117
protein with polyamine oxidase activity	rres127260.v1	AT5G13700	5.85	8.10	9.47	8.00	9.34	0.0008
Pyridoxal-5'-phosphate-dependent enzyme family protein	rres070474.v1	AT5G28237	3.97	8.25	8.98	7.77	8.95	0.0497
Pyridoxal-5'-phosphate-dependent enzyme family protein	rres118374.v1	AT5G28237	2.47	3.91	4.57	3.31	4.60	0.0497
Pyridoxal-5'-phosphate-dependent enzyme family protein	rres110472.v1	AT5G28237	5.87	6.92	7.93	6.73	8.01	0.0117
Pectinacetylesterase family protein	rres037041.v1	AT5G45280	8.04	9.10	9.83	8.87	9.71	0.0294
Protein with similarity to RCD1 but without the WWE domain	rres079531.v1	AT5G62520	2.05	2.88	3.55	2.84	3.68	0.0497
Transport								
Transporter for monosaccharides	rres110493.v1	AT1G08920	2.82	2.88	3.70	2.79	3.30	0.0497
Transporter for monosaccharides	rres045528.v1	AT1G08920	4.11	4.68	5.61	4.37	5.19	0.0297
AKT1, inward rectifying potassium channel	rres095212.v1	AT2G26650	7.44	10.11	10.68	9.68	10.53	0.0296

ABC transporter gene (ABCG22)	rres084719.v1	AT5G06530	6.81	7.27	8.11	6.64	7.84	0.0420
SWEET sucrose efflux transporter family proteins	rres081611.v1	AT5G13170	2.88	2.84	3.56	3.02	3.58	0.0420
Other process								
Galactose oxidase/kelch repeat superfamily protein	rres101432.v1	AT1G67480	4.45	4.96	5.55	5.02	5.46	0.0420
Polyketide cyclase/dehydrase and lipid transport superfamily protein	rres089688.v1	AT4G14500	4.46	5.50	6.21	5.58	6.16	0.0017
O-acyltransferase (WSD1-like) family protein	rres095463.v1	AT1G72110	4.18	8.91	10.34	8.17	10.47	0.0294
Ortholog of sugar beet HS1 PRO-1 2 (HSPRO2);	rres090787.v1	AT2G40000	2.90	3.14	3.87	2.91	3.64	0.0493
Low temperature and salt responsive protein family	rres109929.v1	AT3G05890	2.71	5.68	6.81	5.45	6.62	0.0021
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	rres074815.v1	AT3G44840	6.46	10.57	11.26	10.49	11.04	0.0497
Cold acclimation protein WCOR413-like protein beta form	rres136799.v1	AT3G50830	5.74	4.94	5.93	4.72	6.05	0.0262
SAUR-like auxin-responsive protein family	rres116839.v1	AT3G60690	5.02	5.71	6.31	5.73	6.11	0.0497
S-adenosyl-L-methionine-dependent methyltransferases superfamily protein	rres038199.v1	AT5G04380	2.77	4.46	5.16	3.75	5.64	0.0296
SBP (S-ribonuclease binding protein) family protein	rres053958.v1	AT5G47050	3.27	3.81	5.44	3.86	5.06	0.0205
Unknown	rres092185.v1	AT1G61240	5.62	7.05	7.80	6.85	8.04	0.0021
Unknown	rres072503.v1	AT2G42760	4.12	6.37	7.21	6.14	7.13	0.0008
Unknown	rres086969.v1	AT3G15760	4.18	5.91	7.07	5.56	6.70	0.0329
Unknown	rres105080.v1	AT5G18130	4.42	5.90	6.56	5.71	6.44	0.0262
Unknown	rres063300.v1	AT5G56980	8.78	9.92	10.52	10.00	10.49	0.0400
Unknown	rres071989.v1	AT1G72510	7.79	8.25	8.94	8.40	8.99	0.0493
Unknown	rres067574.v1	AT4G16670	6.28	6.66	7.41	6.77	7.45	0.0283
Unknown	rres082243.v1	AT4G16670	6.81	7.35	8.26	7.50	8.22	0.0166
NA	rres031174.v1	NA	6.19	10.29	10.64	10.18	10.82	0.0497
NA	rres017344.v1	NA	4.69	5.19	5.75	5.24	5.75	0.0403
NA	rres112041.v1	NA	2.94	2.76	3.43	2.63	3.15	0.0432
NA	rres027627.v1	NA	7.13	8.22	9.05	8.41	8.85	0.0400
NA	rres131174.v1	NA	3.13	3.04	4.15	3.21	4.00	0.0117
NA	rres132403.v1	NA	2.67	8.49	9.78	7.88	9.50	0.0309

Table S5. Genes differentially expressed in *Cotesia glomerata*-parasitized *Pieris rapae* or *P. brassicae* compared to unparasitized *P. rapae* or *P. brassicae* treated plants. Relative changes in gene expression in leaves 24h after infestation by either unparasitized *Pieris rapae* (PR), unparasitized *P. brassicae* (PB), *C. glomerata*-parasitized *P. rapae* (PR-CG), *C. glomerata*-parasitized *P. brassicae* (PB-CG), or remained intact as undamaged control (UD). Mean expression ratios are calculated from three biologically independent replicates. AGI, Arabidopsis Genome Initiative. Adjusted P-value indicates effect of parasitism on expression of particular plant gene. NA, not available.

Gene identification	*Brassica* unigene ID	AGI code	UD	PR	PB	PR-CG	PB-CG	Adjusted P-value
Genes with a weaker expression in parasitized compared to unparasitized caterpillars treated plants								
Transcription factor								
Myb-related putative transcription factor	rres096372.v1	AT1G01060	5.77	5.51	5.36	4.81	4.46	0.0491
Protein metabolism/ metabolism								
Ribosomal protein L18ae family	rres080198.v1	AT3G14595	9.07	9.38	9.30	8.74	8.75	0.0491
Dual-targeted protein acts as a pyruvate, orthophosphate dikinase	rres137779.v1	AT4G15530	7.76	6.39	6.31	5.76	5.69	0.0491
Peroxisomal catalase	rres138644.v1	AT4G35090	11.03	9.84	9.61	9.05	9.00	0.0431
Other process								
Unknown, glycine-rich protein	rres036452.v1	AT4G30450	1.75	2.36	2.42	1.81	1.80	0.0438
Unknown	rres110927.v1	AT1G64680	10.18	10.38	10.30	9.68	9.90	0.0431
Genes with a stronger expression in parasitized compared to unparasitized caterpillars treated plants								
Transport								
Maltose transporter	rres065519.v1	AT5G17520	10.55	9.61	9.74	10.28	10.29	0.0431
Maltose transporter	rres075774.v1	AT5G17520	8.66	7.73	7.79	8.47	8.39	0.0431
Unknown, contains InterPro DOMAIN/s: Nucleic acid-binding	rres109266.v1	AT5G19300	5.50	5.61	5.73	6.20	6.16	0.0431
Germin-like protein	rres101760.v1	AT5G20630	7.33	5.13	5.53	6.94	6.54	0.0438
Protein metabolism/ metabolism								
ATP-dependent caseinolytic (Clp) protease/crotonase family protein	rres054966.v1	AT1G06550	5.45	5.78	6.00	6.68	6.63	0.0438
Other process								
Protein predicted to act as a carboxylesterase	rres092888.v1	AT1G33990	4.97	5.20	5.32	6.09	6.02	0.0438
Late embryogenesis abundant protein (LEA) family protein	rres037668.v1	AT3G62580	2.32	2.53	2.62	3.36	3.39	0.0438
YLMG2, plasma membrane	rres019962.v1	AT5G21920	7.18	6.50	7.02	7.99	7.86	0.0431
ChaC-like family protein	rres132261.v1	AT5G26220	2.36	3.70	3.72	5.03	4.75	0.0431
Unknown	rres110504.v1	AT1G10657	6.66	5.87	5.90	6.88	6.67	0.0431
NA	rres013651.v1	NA	7.69	7.28	7.00	8.53	7.90	0.0438

Chapter 5

Labial saliva of parasitized caterpillars affects plant-mediated indirect species interactions

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Abstract

Plant traits mediate complex indirect interaction networks in food webs. For example, parasitic wasps affect the phenotype of their host caterpillar that induces changes in plant traits that influence other organisms interacting with the plant. Previous studies showed that parasitized *Pieris brassicae* caterpillars induce a different response in their *Brassica* food plant than healthy caterpillars, which allows the hyperparasitoid *Lysibia nana* to locate its parasitoid host and alters the interaction of *Brassica* plants with a subsequently colonizing moth, *Plutella xylostella.* These studies suggested that caterpillar oral secretions play a major role in driving the plant-mediated interaction network, because parasitoids affected the composition of their host's oral secretions. However, oral secretions are complex mixtures of substances with different origins and it is unknown which components are affected by parasitism. In this study, we surgically removed caterpillar labial salivary glands to address the role of labial saliva in plant-mediated multitrophic interactions by combining insect behavioural studies and plant volatile headspace analysis. In addition, using transcriptome sequencing, we studied the parasitism-induced physiological changes in caterpillar labial salivary glands. Our results show that *P. xylostella* and *L. nana* cannot distinguish between plants induced by ablated unparasitized or parasitized caterpillars and respond to plants induced by ablated caterpillars similarly as to undamaged control plants. We found differences in the blend of plant volatiles induced by ablated or mock-treated caterpillars. Moreover, transcripts of genes encoding the herbivore-associated elicitors β-glucosidase and glucose oxidase were differentially regulated in salivary glands of parasitized caterpillars compared to unparasitized caterpillars. Our study shows that herbivore labial saliva plays an important role in plant-herbivore interactions. The extended phenotypes of parasitoids as expressed in the changes in the saliva of their herbivorous host strongly alter the plant trait-mediated indirect interactions.

Keywords: trait-mediated indirect interactions; extended phenotype; labial salivary glands; herbivore saliva; parasitism; hyperparasitoid; *Pieris brassicae.*

Introduction

Trait-mediated indirect species interactions are a major component of community organisation (Werner & Peacor 2003). These indirect interactions occur when one species affects a second species via the outcome of its direct interaction with a third species. In terrestrial ecosystems, plant phenotypic responses to biotic and abiotic conditions strongly mediate interactions with its associated community members (Price *et al.* 1980; Sultan 2000; Loreto & Schnitzler 2010). Biotically or abiotically induced plant traits are perceived by a broad range of other community members, from neighbouring plants (Callaway *et al.* 2003; Karban 2008) to below-ground organisms (Rasmann *et al.* 2005; Robert *et al.* 2012; Pangesti *et al.* 2013), from herbivorous insects (Karban & Agrawal 2002; Poelman *et al.* 2009) to natural enemies of herbivores (Vet & Dicke 1992; Heil 2008; Gols & Harvey 2009), even to the organisms at the fourth tropic level (Poelman *et al.* 2012; Zhu *et al.* 2015). Thus, plant trait-mediated interactions may significantly affect the composition and dynamics of plant-associated communities (van Zandt & Agrawal 2004; Ohgushi *et al.* 2011; Utsumi 2011).

Because plants strongly respond to insect herbivory by mobilizing their defences, herbivorous insects profoundly affect the plant phenotype and its interactions with other community members (van Zandt & Agrawal 2004; Kessler & Halitschke 2007; Dicke & Baldwin 2010). In response to herbivore attack, plants have evolved a set of sophisticated direct and indirect defences whose induction may be specific for the feeding guild (chewing vs sap sucking), specialisation (generalist vs specialist) and even species identity of the attacking herbivore (Heil 2008; Howe & Jander 2008; Bari & Jones 2009; Broekgaarden *et al.* 2011; Ali & Agrawal 2012). To activate these specific defence responses, plants need to recognize herbivore attack by its feeding pattern and to perceive chemical cues released by herbivores in the form of herbivore-associated elicitors or herbivore-associated molecular patterns (HAMPs) (Bonaventure 2012). The elicitors thereby activate signal-transduction pathways regulated by gene transcriptional responses and phytohormones that modulate herbivory-induced responses in plants (Erb *et al.* 2012; Pieterse *et al.* 2012). Thus far, several herbivore-associated elicitors have been identified in herbivore oral secretions that are closely associated with feeding by the herbivores (Vadassery *et al.* 2012). These elicitors are diverse in their molecular structures, including enzymes (e.g. glucose oxidase, β-glucosidase) (Mattiacci *et al.* 1995; Musser *et al.* 2002), fatty acid–amino acid conjugates (Alborn *et al.* 1997), sulphur-containing fatty acids (caeliferins) (Alborn *et al.* 2007), fragments of cell walls (e.g. oligogalacturonides) (Doares *et al.* 1995; Bergey *et al.* 1999), as well as peptides released from digested plant proteins (e.g. inceptins; proteolytic fragments of the chloroplastic ATP synthase γ-subunit) (Schmelz *et al.* 2006).

The indirect trait-mediated interactions that arise from herbivore-plant interactions may also be part of a network of trait-mediated interactions across multiple trophic levels in a food web. One such an example is the interaction network mediated by herbivore-induced plant volatiles (HIPVs) that allow hyperparasitoids to locate their parasitoid host (Poelman *et al.* 2012; Zhu *et al.* 2015). Hyperparasitoids are parasitic wasps at the fourth trophic level within the plant-associated insect community. Primary hyperparasitoids oviposit in the larvae of their parasitoid host while these are still developing inside a herbivore host, whereas secondary hyperparasitoids attack the pupae of their parasitoid host (Sullivan 1987). Remarkably, hyperparasitoids are able to perceive changes in the blend of HIPVs induced by healthy versus parasitized caterpillars, and prefer HIPVs emitted by plants damaged by parasitized caterpillars over those emitted by plants damaged by healthy caterpillars (Poelman *et al.* 2012; Zhu *et al.* 2015). In addition, the presence of parasitoid larvae inside a herbivore host changes the expression patterns of herbivore-induced plant defence-related genes and the interactions between the plant and subsequently colonizing herbivores (Poelman *et al.* 2011b). This is remarkable, because parasitoid larvae do not feed on plants; they even hardly make direct contact with plants. Their effect on plants is mediated by their herbivore host that in turn extends the effects of the parasitoids to interact with the food plant in a trait-mediated interaction network that may further affect oviposition and foraging preferences of subsequently colonizing moths, parasitoids and hyperparasitoids (Fatouros *et al.* 2005; Poelman *et al.* 2011a; Poelman *et al.* 2011b; Poelman *et al.* 2012).

It has been recognized that different herbivore-associated organisms (HAOs), including parasitoid larvae, largely affect the behavioural and physiological conditions of their herbivore host (Hughes *et al.* 2012; Zhu *et al.* 2014a). While developing in their caterpillar host, parasitoid larvae induce physiological changes in the host, resulting in altered composition of host oral secretions (Poelman *et al.* 2011b). Therefore, it is likely that parasitism affects the composition of the elicitors in caterpillar oral secretions, and then influences the herbivore-induced plant response. However, caterpillar oral secretion is a complex mixture, consisting of saliva, foregut substances and diverse micro-organisms. Thus, the changes that parasitoid larvae induce in herbivore saliva that subsequently affect the interaction network and that allow hyperparasitoids to locate the parasitoids, remains to be elucidated. Because caterpillar oral secretions are a mixture of compounds derived from several tissues, the effects of parasitism on physiological changes in the herbivore host should be investigated in a host tissue-specific manner.

In this study, we specifically addressed how parasitoid larvae developing inside a herbivore indirectly affect plant responses to herbivory and plant volatile-mediated multitrophic interactions, using a study system including organisms from four

trophic levels in a *Brassica oleracea* plant-based food web. Here, we focused on the labial salivary glands that have a prominent role in caterpillar feeding and their importance for plant defence responses has been demonstrated by using an ablation technique (Musser *et al.* 2002; Musser *et al.* 2006; Musser *et al.* 2012). The effect of labial saliva on induced plant responses that affect plant-mediated multitrophic interactions were investigated for plant-odour-based host location behaviour of the secondary hyperparasitoid *Lysibia nana*. Moreover, we used the Diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) as an indicator of herbivore-induced plant phenotypic changes as it is sensitive to phenotypic changes in induced plants (Bruinsma *et al.* 2010; Poelman *et al.* 2011b). It is known that *P. xylostella* prefers to oviposit on plants that have been previously damaged by other herbivores, whereas plants damaged by parasitized caterpillars are less preferred for oviposition in comparison to plants induced by unparasitized caterpillars (Poelman *et al.* 2011b). Subsequently, the differences in HIPV blends induced by mock-treated caterpillars and caterpillars with salivary glands ablated were compared. Finally, a transcriptome sequencing approach was used to study gene expression differences in labial salivary glands between unparasitized and parasitized herbivores. We discuss how the presence of parasitoids exhibits an extended phenotype through their effects on herbivore saliva that subsequently affects different plant-insect interactions.

Materials and methods

Plants and insects

The wild *Brassica oleracea* population “Kimmeridge” (seeds were collected in Dorset, UK, 50°360N, 2°070W) was used in this study since this *Brassica* population has been shown to differentially respond to feeding by healthy and parasitized herbivores (Zhu *et al.* 2015). Plants were grown under conditions described in Zhu *et al.* (2015). Five-week-old plants were used in the experiments.

The two herbivore species, the large cabbage white *Pieris brassicae* L. (Lepidoptera: Pieridae) and the Diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae), and parasitoid species, the larval parasitoid *Cotesia glomerata* L. (Hymenoptera: Braconidae) and the hyperparasitoid *Lysibia nana* Gravenhorst (Hymenoptera: Ichneumonidae) used in this study were originally collected from field sites near Wageningen University, the Netherlands. They were reared on (hosts on) cultivated cabbage plants (*B. oleracea* var. *gemmifera* cv. Cyrus) in glasshouse compartments (22 ± 1 °C, 50-70% relative humidity and 16:8 h L:D photoperiod). To prepare parasitized caterpillars for plant induction treatments, individual first-instar *P. brassicae* larvae were exposed to a single female *C. glomerata*, which was allowed to parasitize the larva in a glass vial. The caterpillar was considered to be parasitized when the wasp

had inserted her ovipositor in the caterpillar for at least five seconds. To avoid effects caused by depletion of the parasitoids' egg load, no more than ten caterpillars were offered to a single female parasitoid. The parasitized caterpillars were reared until the fifth instar when they were used for induction treatments. The hyperparasitoid *Lysibia nana* was recovered from field-collected *C. glomerata* cocoons and was reared on *C. glomerata* cocoons in the absence of plant- and herbivore-derived cues.

Protocol for ablation of *P. brassicae* labial salivary glands

Ablation of labial salivary glands was performed on both unparasitized and *C. glomerata*-parasitized *P. brassicae* caterpillars when they reached the second-day of their fifth larval instar and followed methods described in Musser *et al.* (2006). In brief, the selected unparasitized and parasitized caterpillars were contained in separate 7-inch Petri dishes and sedated by chilling on ice for 15 min. Then, one single caterpillar was transferred to a dissection plate that was filled with an ice-cold autoclaved solution of phosphate-buffered saline (PBS; Oxoid, Hampshire, UK). While the caterpillar was submerged in PBS solution, the second abdominal segment between the true legs and prolegs was held from the dorsal side of the caterpillar using forceps. Subsequently, a miniscule incision was made in the cuticle revealing the pair of labial salivary glands. With a forceps, the complete labial salivary glands were gently removed from the body cavity. For parasitized caterpillars, larvae of *C. glomerata* occasionally emerged from the incision. Therefore, only those caterpillars that had no more than three out of a brood size of 15-30 parasitoid larvae slipping out of the incision were included in the study. After the ablation of the salivary glands, the caterpillar was rinsed with distilled water, dried with tissue paper and transferred to a new Petri dish supplied with a fresh *B. oleracea* leaf. The caterpillar was allowed to recover from the surgery in the Petri dish for three hours. Caterpillars that within these three hours started feeding on the plant leaf were selected for subsequent plant induction. Mock-treated unparasitized and parasitized caterpillars were subjected to the same protocol, including the incision, but the labial salivary glands were not removed from the body cavity of the caterpillar. To ensure that ablated caterpillars fed similar amounts of leaf tissue as mock treated caterpillars, we quantified the amount of leaf damage for 10 plants for each herbivore-induced treatment, using a transparent plastic sheet with 1 mm^2 grid.

Y-tube olfactometer assays

We offered females of *L. nana* two-choice tests for combinations of five plant induction treatments in a Y-tube olfactometer setup as described by Takabayashi and Dicke (1992). The wild *B. oleracea* plants were treated with two fifth-instar caterpillars for 24 hours: 1) *P. brassicae* caterpillars with intact labial salivary glands (PB-S+); 2) *P. brassicae* caterpillars with ablated labial salivary glands (PB-S-); 3) *C. glomerata*

parasitized *P. brassicae* caterpillars with intact labial salivary glands (PB-CG-S+); 4) *C. glomerata* parasitized *P. brassicae* caterpillars with ablated labial salivary glands (PB-CG-S-); or 5) were left untreated serving as the undamaged control (UD). In our previous work, we have shown that *L. nana* prefers plant volatiles induced by unparasitized and parasitized caterpillars over undamaged plants, and that volatiles from plants damaged by parasitized caterpillars are preferred over those from plants damaged by unparasitized caterpillars (Zhu *et al.* 2015). For clarity of the results obtained in the current study, we included these results as reference in Figure 1a. In the current study, we tested whether parasitization of the caterpillars affected the composition of compounds in the labial salivary gland to the extent that this resulted in differential effects on the elicitation of plant response. We first offered *L. nana* plant volatiles induced by unparasitized or parasitized *P. brassicae,* both with ablated labial salivary glands to test whether this hyperparasitoid could still discriminate these treatments. Subsequently, we tested *L. nana* attracttion to plant volatiles induced by mock-treated caterpillars or caterpillars from which the labial salivary glands had been ablated within the same category (unparasitized or parasitized). Finally, we tested preferences of *L. nana* for plant volatiles released by undamaged control plants versus plant volatiles induced by unparasitized or parasitized *P. brassicae* caterpillars with ablated labial salivary glands, to test whether hyperparasitoids respond to plant volatiles induced by caterpillars without labial salivary glands. For each pairwise comparison, 70 *L. nana* females were tested. The Y-tube olfactometer assays followed the procedures described in Zhu *et al.* (2015).

Plutella xylostella oviposition assays

Plants were subjected to the five induction treatments as described above and then used for oviposition preference assays for Diamondback moths following methods described in Poelman *et al.* (2011). Shortly before the oviposition assay, we excised the leaves from the plants and directly placed them with the petioles in glass vials filled with tap water. We matched two leaves from different induction treatments with similar size and caterpillar feeding damage. The leaf pair was placed in a plastic cylinder (diameter 145 mm, height 220 mm), and then one male and one female Diamondback moth were released in the cylinder. The female moths were allowed to oviposit overnight. The number of eggs on each leaf was counted the next morning. Diamondback moths are known to be sensitive to subtle changes in plant phenotype and prefer to oviposit on plants that were previously damaged by other herbivores (Poelman *et al.* 2008a). The moths prefer to lay eggs on plants damaged by healthy *Pieris* caterpillars over parasitized caterpillars (Figure 2a; Poelman *et al.* 2011b). Therefore, here, we first tested whether the moth is able to discriminate plants induced by unparasitized or parasitized caterpillars with salivary glands ablated. Thereafter, we tested whether *P. xylostella* discriminate between plants induced by mock-treated

caterpillars or caterpillar with labial salivary glands removed (PB-S+ vs PB-S-; and PB-CG-S+ vs PB-CG-S-). Finally, we tested whether *P. xylostella* exhibit differential oviposition to undamaged control plants and plants damaged by ablated unparasitized (UD vs PB-S-) or parasitized (UD vs PB-CG-S-) *P. brassicae*.

Plant volatile headspace collection and analysis

To characterize the effects of labial saliva of *P. brassicae* in emission of HIPVs in wild *B. oleracea* plants, we collected 10 plant volatile samples for each plant treatment. We treated plants followed procedures described above. The subsequent plant volatile collections were followed procedures described in Zhu *et al.* (2015).

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 releasing of the volatiles, each sample was dry-purged under a flow 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 adsorbent thermally using Ultra 50:50 thermal desorption unit (Markes, Llantrisant, UK) at 250 °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 0 °C. Once the desorption process is completed, volatile compounds were released from the cold trap by ballistic heating at 40 °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 0.25 ㎜ F.T. with 5 m build in guard column (Phenomenex, Torrance, CA, USA)], in a splitless for further separation. The GC oven temperature was initially held at 40 °C for 2 min and was immediately raised at 6 °C min^{-1} to a final temperature of 280 °C, where it was kept for 4 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. MS transfer line and ion source were set at 275 and 250 °C, respectively. Tentative identification of compounds was based on comparison of mass spectra with those in the NIST 2005 and Wageningen Mass Spectral Database of Natural Products MS libraries as well as experimentally obtained linear retention indices (LRI).

Labial salivary glands extraction and RNA isolation

To study the tissue-specific transcriptional differences in unparasitized and *C. glomerata* parasitized caterpillars, labial salivary glands of the two types of caterpillar were extracted following the ablation procedure described above. We pooled 15 pairs of labial salivary glands per sample. After extraction, samples were immediately flash-frozen in liquid nitrogen. Total RNA was extracted from each of the labial salivary gland samples (4 samples from unparasitized *P. brassicae* and 4 samples from *C. glomerata* parasitized

P. brassicae larvae) using the innuPREP RNA Mini Isolation Kit (Analytik Jena, Jena, Germany) following the manufacturers' guidelines. The integrity of the RNA was verified using an Agilent 2100 Bioanalyzer and a RNA 6000 Nano Kit (Agilent Technologies, Palo Alto, CA). The quantity as well as OD 260/280 and 260/230 values of the isolated RNA samples were determined using a Nanodrop ND-1000 spectrophotometer.

Illumina sequencing and transcriptome assembly

Tissue-specific transcriptome sequencing of eight RNA pools was carried out on an Illumina HiSeq2500 Genome Analyzer platform using paired end (2 x 100 bp) read technology with RNA fragmented to an average of 150 nucleotides. Library construction and sequencing was performed by the Max Planck Genome Center Cologne, Germany (http://mpgc.mpipz.mpg.de/home/). 1 µg of total RNA each was used for generating TruSeq RNA libraries and mRNA enrichment was performed. Approximately 40 million reads per biological replicate and per treatment were obtained. Quality control measures, including filtering high-quality reads based on the score given in fastq files, removing reads containing primer/adaptor sequences and trimming read length, were carried out using CLC Genomics Workbench v7.1 (http://www.clcbio.com). The *de novo* transcriptome assembly (TA) was carried out using CLC Genomics Workbench software v7.1 (http://www.clcbio.com) by comparing an assembly with standard settings and two additional CLC-based assemblies with different parameters, selecting the presumed optimal consensus transcriptome according to published details (Vogel *et al.* 2014). Any conflicts among the individual bases were resolved by voting for the base with highest frequency. Contigs shorter than 200 bp were removed from the final analysis. The resulting final *de novo* reference TA (backbone) contained 24,054 contigs with a N50 contig size of 2432 bp and a maximum contig length of 22092 bp.

Homology searches and annotation

BLASTx and BLASTn homology searches with our unique sequences were conducted on a local server using the National Center for Biotechnology Information (NCBI) blastall program. First, sequences were searched against the NCBI NR protein database using an E-value cut-off of 10^{-3} to find predicted polypeptides with a minimum length of 15 amino acids. Second, sequences with no BLASTx hits were used as queries in a BLASTn search against an NCBI NR nucleotide database with an E-value cut-off of 10^{-10}. Blast results were imported as xml files and further processed using the BLAST2GO-PRO software suite (www.blast2go.de) (Conesa *et al.* 2005). Functional annotations were assigned to the *P. brassicae* TA contigs using a sequential strategy based on gene ontology (GO) terms (www.geneontology.org), InterPro terms (InterProScan, EBI), enzyme classification (EC) codes and KEGG metabolic pathways (Kyoto Encyclopedia of Genes and Genomes). Enzyme classification codes and KEGG metabolic pathway annotations were generated from the direct mapping of GO terms to their enzyme

code equivalents. Finally, InterPro searches were carried out remotely against the InterProEBI web server. Enrichment analyses were carried out by comparing the GO-annotations from each differentially expressed contig subset (test sets) with the complete TA contig set (reference set) by running a two-tailed Fisher's exact test using the appropriate Blast2GO web application (http://www.blast2go.com/webstart/makeJnlp.php) with false discovery rate (FDR) correction for multiple testing and a P-value of 0.05. The Blast2GO web application was configured to access the local GO database previously used to assign GO terms.

Digital gene expression analysis

Digital gene expression analysis was carried out by using QSeq Software (DNAStar Inc.) to remap the Illumina reads from all eight samples onto the reference backbone and then counting the sequences to estimate expression levels using previously described parameters for read mapping and normalization (Vogel *et al.* 2014). For read mapping, we used the following parameters: n-mer length = 25; read assignment quality options required at least 25 bases (the amount of mappable sequence as a criterion for inclusion) and at least 90% of bases matching (minimum similarity fraction, defining the degree of preciseness requires) within each read to be assigned to a specific contig; maximum number of hits for a read (reads matching a greater number of distinct places than this number are excluded) = 10; n-mer repeat settings were automatically determined and other settings were not changed. Biases in the sequence datasets and different transcript sizes were corrected using the RPKM algorithm (reads per kilobase of transcript per million mapped reads) to obtain correct estimates for relative expression levels. To control for the effect of global normalization using the RPKM method, we also analyzed a number of highly conserved housekeeping genes frequently used as control genes in qPCR analysis. These controls included several genes encoding ribosomal proteins (rpl3, rpl5, rpl7a, rps3a, rps5, rps8, rps18 and rps24), elongation factor 1alpha and eukaryotic translation initiation factors 4 and 5. The corresponding genes were inspected for overall expression levels across samples and were found to display expression level differences (based on RPKM values) lower than 1.3-fold between samples, indicating they were not differentially expressed and validating them as housekeeping genes. Hierarchical clustering was performed with the QSeq software using the Euclidean distance metric and using the Centroid Linkage method.

Statistical analysis

The preferences by *L. nana* for HIPVs were analysed using two-tailed binomial tests (SPSS 19; Chicago, IL, USA). The oviposition preferences of *P. xylostella* were analysed using *Wilcoxon matched-pair signed-rank tests* (SPSS 19; Chicago, IL, USA).

Volatile emission multivariate data analysis was carried out using principal component analysis (PCA) and projection to latent structures-discriminant analysis (PLS-DA; PCA and PLS-DA modules of SIMCA-P 12.0.1, Umetrics, Umeå, Sweden). The measured peak areas for the volatile blends in the different treatments were log-transformed, mean centred and scaled to unit variance before being analysed using PCA and PLS-DA. The results of the PLS-DA analysis are visualized in score plots and loading plots. The score plots reveal the sample structure according to the model components. The loading plots display the contribution of the variables to the components and the relationships among the variables. Mann-Whitney U-tests were used to test the differences in emission of individual volatile compounds that were tentatively identified in the headspace of herbivore-induced wild *B. oleracea* plants (SPSS 19; Chicago, IL, USA).

Results

Effects of ablation of labial salivary glands on caterpillar performance

We did not find apparent reduction in food consumption of ablated caterpillars compared to mock-treated caterpillars (Student's *t*-tests; for unparasitized caterpillars, $F = 1.197$, $df = 18$, $P = 0.471$; for parasitized caterpillars, $F = 1.202$, $df = 18$, $P = 0.118$). After the experiments, the ablated unparasitized caterpillars successfully pupated and eclosed as adult butterflies. For ablated parasitized caterpillars, fully grown parasitoid larvae eventually emerged and pupated. However, ablated caterpillars did not produce silk.

Reponses of hyperparasitoids to HIPVs induced by caterpillars with labial salivary glands removed

In Y-tube ofactometer assays, approximately 70% of the hyperparasitoids *L. nana* made their final choices. Our previous work showed that *L. nana* preferred volatiles from plants damaged by herbivores (for both unparasitized and parasitized caterpillars) over undamaged control plants (Figure 1a; binomial tests, $P < 0.01$; for both PB and PB-CG). In addition, plant volatiles induced by *C. glomerata*-parasitized *P. brassicae* were more attractive than those induced by unparasitized caterpillars (Figure 1a; binomial test, $P < 0.01$). However, in the current study the hyperparasitoid preferences for caterpillar-damaged plants were lost when the caterpillar salivary glands were removed. *L. nana* showed equal preferences to HIPVs induced by ablated unparasitized or *C. glomerata*-parasitized caterpillars (Figure 1b; binomial test; PB-S- vs PB-CG-S-: $P = 0.888$). Moreover, *L. nana* did not discriminate HIPVs released by plants induced by mock-treated unparasitized *P. brassicae* from HIPVs released by plants induced by ablated *P. brassicae* (Figure 1b; binomial tests; PB-

S- vs PB-S+: P = 0.092). The wasps showed stronger attraction to HIPVs induced by mock-treated parasitized caterpillars than those induced by ablated parasitized caterpillars (Figure 1b; binomial test; PB-CG-S+ vs PB-CG-S-: P < 0.01). Furthermore, when hyperparasitoids were offered plant volatiles induced by ablated caterpillars (unparasitized and parasitized) and undamaged control plants, they showed equal preferences for their volatiles (Figure 1b; binomial tests; UD vs PB-S-: P = 0.56; UD vs PB-CG-S-: P = 0.065).

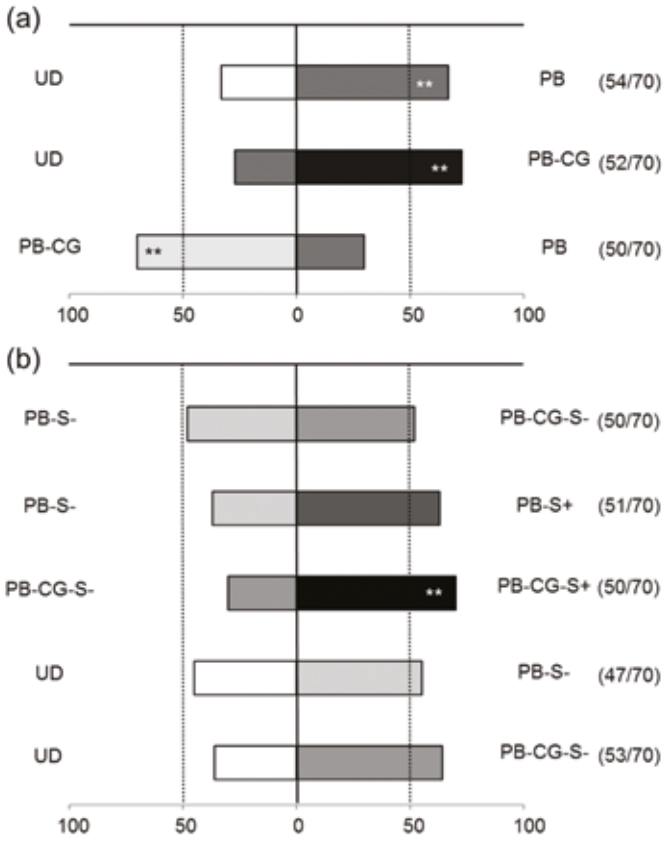

Figure 1. Preference of hyperparasitoids (*L. nana*) for herbivore-induced plant volatiles in two-choice Y-tube olfactometer tests, (a) data obtained from a previous study (Zhu *et al.* 2015), pair-wise comparisons between undamaged control plants (UD), *P. brassicae*-damaged plants (PB), and plants damaged by *C. glomerata*-parasitized *P. brassicae* caterpillars (PB-CG); (b) behavioural bioassays performed in the present study, comparing undamaged control plants (UD), plants damaged by ablated *P. brassicae* caterpillars (PB-S-), plants damaged by mock-treated *P. brassiace* caterpillars (PB-S+), plants damaged by ablated *C. glomerata*-parasitized *P. brassicae* caterpillars (PB-CG-S-), and plants damaged by mock-treated *C. glomerata*-parastized *P. brassicae* caterpillars (PB-CG-S+. Numbers between brackets indicate the number of wasps that made a choice within 10 min from the start of the experiment out of the total number of wasps tested. **P < 0.01.

Oviposition preferences of Diamondback moths to plants induced by caterpillars with labial salivary glands removed

In two-choice tests, Diamondback moths preferred to oviposit on plants damaged by mock-treated unparasitized *P. brassicae* caterpillars over undamaged control plants (Figure 2b; Wilcoxon's matched-pairs signed-ranks test, $P < 0.001$), confirming earlier findings that the moths prefer healthy or parasitized herbivore damage over undamaged plants (Figure 2a) (Poelman *et al.* 2011b). The preference for plants damaged by parasitized caterpillars over unparasitized caterpillars (Figure 2a), was lost when salivary glands of both caterpillars were ablated (Figure 2b; Wilcoxon's matched-pairs signed-ranks test, PB-S- vs PB-CG-S-: $P = 0.741$). Furthermore, Diamondback moths oviposited fewer eggs on plants induced by ablated *P. brassicae* caterpillars compared to plants induced by mock-treated *P. brassicae* (Figure 2b; Wilcoxon's matched-pairs signed-ranks test, PB-S- vs PB-S+: $P = 0.001$). Similarly, the moths laid more eggs on plants induced by mock-treated parasitized *P. brassicae* than plants induced by ablated parasitized *P. brassicae* caterpillars (Figure 2b; Wilcoxon's matched-pairs signed-ranks test, PB-CG-S+ vs PB-CG-S-: $P = 0.032$). Even more so, the moths did not differentially oviposit on undamaged control plants and plants induced by ablated unparasitized or parasitized caterpillar (Figure 2b; Wilcoxon's matched-pairs signed-ranks test; UD vs PB-S-: $P = 0.129$; UD vs PB-CG-S-: $P = 0.181$).

Plant volatile analysis

In total, 50 volatile compounds were tentatively identified across all five experimental plant treatments. Apart from the absence of (*E*)-2-butenenitrile in undamaged control plants, there were no other qualitative differences in the composition of volatile blends among treatments (Table 1). A multivariate analysis that included all sampled plant treatments resulted in a model with one significant principle component (Figure 3a; PLS-DA, $R^2X = 0.195$, $R^2Y = 0.13$, $Q^2 = 0.064$). In this model, a total of 19 compounds had VIP (variable importance in the projection) values > 1 (Figure 3b), which were the most important compounds that differentiated the volatile blends. These compounds include nine monoterpenes, two sesquiterpenes, two nitriles, two ketones, two esters, one alcohol, and one unknown compound (Table 1). The total HIPV emission rates showed significant differences among treatments (Table 1; ANOVA, df = 4, $F = 3.861$, $P = 0.009$). Plants induced by mock-treated unparasitized or parasitized *Pieris* caterpillars released a higher amount of plant volatiles than undamaged control plants, whereas total HIPV emission rate of plants induced by ablated caterpillars did not significantly differ from the emission rate of undamaged control plants.

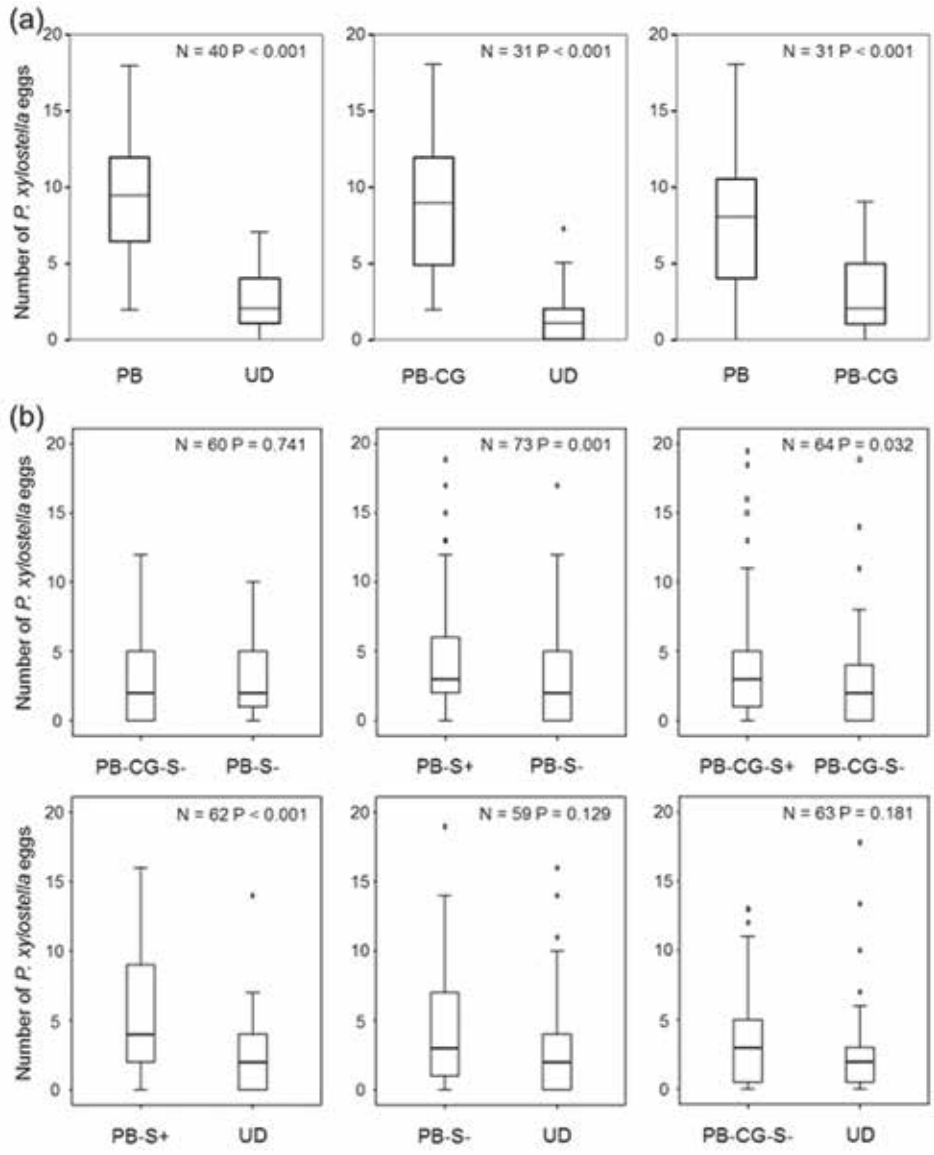

Figure 2. Oviposition preference of Diamondback moths (*P. xylostella*) for *B. oleracea* leaves induced by feeding damage of *P. brassicae* caterpillars. Treated leaves were offered in two-choice tests, (a) data obtained in a previous study (Poelman *et al.* 2011), pair-wise comparisons between undamaged control leaves (UD), *P. brassicae*-damaged leaves (PB), and leaves damaged by *C. glomerata*-parasitized *P. brassicae* caterpillars (PB-CG); (b) tests performed in the present study, comparing undamaged control plants (UD), plants damaged by ablated *P. brassicae* caterpillars (PB-S-), plants damaged by mock-treated *P. brassiace* caterpillars (PB-S+), plants damaged by ablated *C. glomerata*-parasitized *P. brassicae* caterpillars (PB-CG-S-), and plants damaged by mock-treated *C. glomerata*-parastized *P. brassicae* caterpillars (PB-CG-S+).

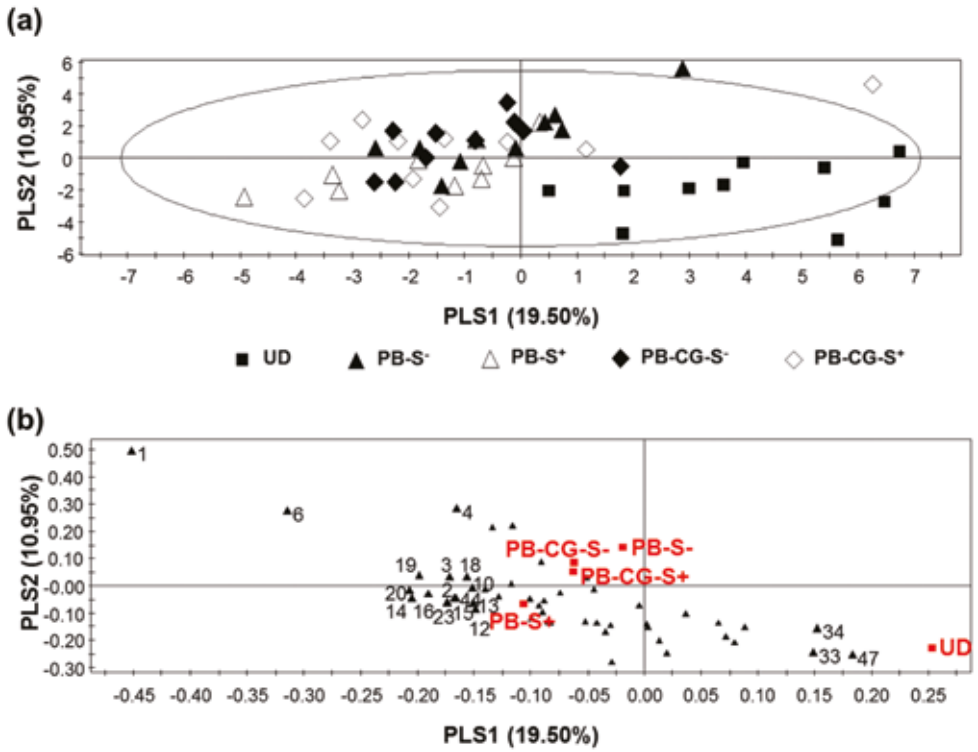

Figure 3. Projection to latent structures–discriminant analysis (PLS-DA; n=10) of the blends of volatile compounds emitted by plants in response to either ablated *P. brassicae* caterpillars (PB-S-), mock-treated *P. brassiace* caterpillars (PB-S+), ablated *C. glomerata*-parastized *P. brassicae* caterpillars (PB-CG-S-), mock-treated *C. glomerata*-parastized *P. brassicae* caterpillars (PB-CG-S+) or plants that had remained undamaged (UD). The score plot (A) visualizes the structure of the samples according to the first two principal components with the explained variance in brackets. The Hotelling's T^2 ellipse confines the confidence region (95%) of the score plot. The loading plot (B) defines the contribution of each of the volatile compounds to the first two principal components. Numbers indicate the identity of the compounds that have variable importance in the projection (VIP) values larger than 1. Compound identities and their respective numbers are presented in Table 1.

Pairwise comparison by PLS-DA for plant volatiles induced by mock-treated and ablated unparasitized *P. brassicae* revealed a model with one significant principle component (Figure 4a; PLS-DA, R^2X = 0.223, R^2Y = 0.408, Q^2 = 0.08). Among the 21 compounds that had VIP values > 1 (Figure 4a), three compounds showed higher emission by plants that were induced by mock-treated unparasitized caterpillars, which were 3-methylbutanenitrile, (*E*)-4,8-dimethyl-1,3,7-nonatriene (DMNT) and (*E*,*E*)-α-farnesene ((Mann–Whitney *U* tests, P = 0.041, P = 0.041, and P = 0.049, respectively).

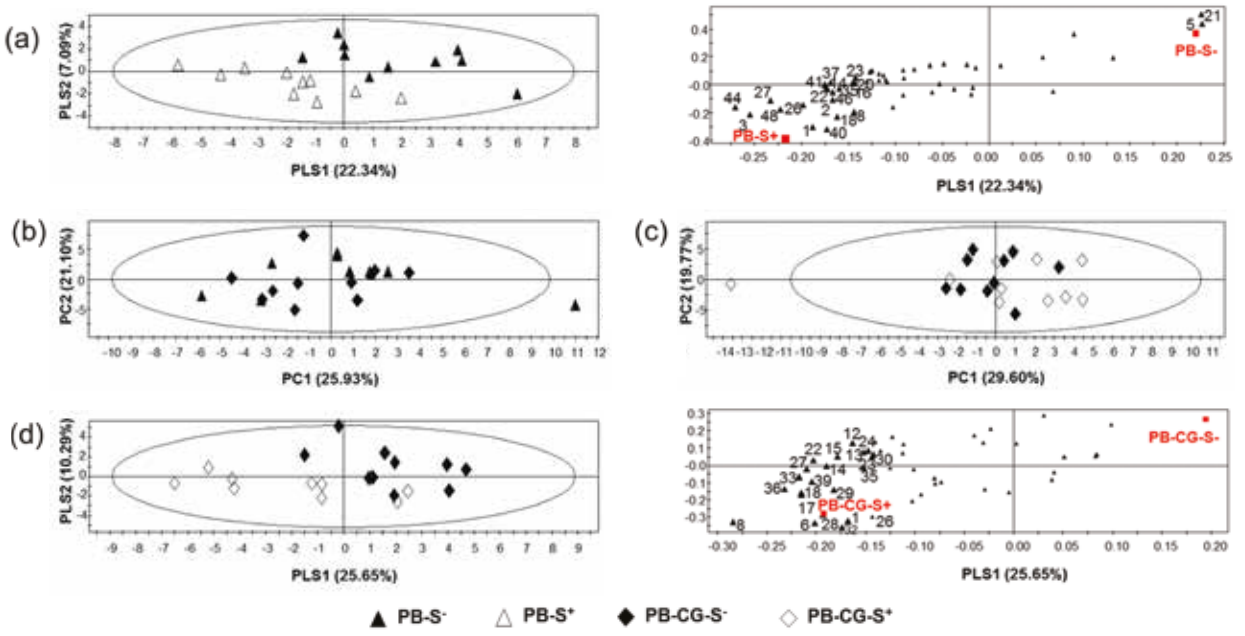

Figure 4. Plant headspace analysis. (a) Projection to latent structures–discriminant analysis (PLS-DA, n=10) of the blends of volatile compounds emitted by plants in response to either ablated *P. brassicae* caterpillars (PB-S-) or mock-treated *P. brassiace* caterpillars (PB-S+). The score plot (left) visualizes the structure of the samples according to the first two principal components with the percentage explained variance in brackets. The loading plot (right) defines the contribution of each of the volatile compounds to the first two principal components. (b) Score plot of principal component analysis (PCA; n=10) of the volatile blends of plant damaged by either ablated *P. brassicae* caterpillars (PB-S-) or ablated *C. glomerata*-parastized *P. brassicae* caterpillars (PB-CG-S-). These two blends overlapped in the multivariate space (the PLS-DA analysis did not result in a significant model). (c) Score plot of PCA (n=10) of the volatile blends of plants damaged by either ablated *C. glomerata*-parastized *P. brassicae* caterpillars (PB-CG-S-) or mock-treated *C. glomerata*-parastized *P. brassicae* caterpillars (PB-CG-S+). These two blends overlapped in the multivariate space (the PLS-DA analysis did not result in a significant model). (d) PLS-DA analysis of the volatile blends of plant damaged by either PB-CG-S- (n=10) or PB-CG-S+ (n=9). The score plot (left) visualizes the structure of the samples according to the first two principal components with the percentage explained variance in brackets. The loading plot (right) defines the contribution of each of the volatile compounds to the first two principal components. Numbers in the loading plots of PLS-DA indicate the identity of the compounds that have variable importance in the projection (VIP) values larger than 1. Compound identities and their respective numbers are presented in Table 1.

Table 1. Volatile compounds tentatively identified in the headspace of wild *Brassica oleracea* 'Kimmeridge' plants. Volatile emissions are given as mean peak area (SE) per gram fresh weight of plant divided by 10^4. Variable importance in the projection (VIP) values for the projection to latent structures–discriminant analysis are given. VIP values larger than 1 are shown boldfaced. Differences among treatments for compounds based on Mann–Whitney U pairwise comparisons are indicated with superscript letters.

No.	Compound	Class	UD^x (n = 10)	PB-S-^{x} (n = 10)	PB-S+^{x} (n = 10)	PB-CG-S-^{x} (n = 10)	PB-CG-S+^{x} (n = 10)	VIP score
1	(*E*)-2-butenenitrile	Nitrile	-^{a}	22.9 (6.5)^b	61.8 (22.3)^b	22.9 (7.2)^b	85.6 (37.9)^b	**3.20**
2	1-penten-3-ol	Alcohol	19.3 (6.7)^a	78.1 (26.1)^{ab}	215.2 (99.0)^b	26.8 (8.3)^a	80.6 (37.4)^{ab}	**1.19**
3	3-pentanone	Ketone	6.5 (1.5)^a	10.9 (3.1)^{ab}	32.5 (7.4)^b	8.9 (2.3)^a	23.8 (12.6)^{ab}	**1.21**
4	2-methylbutanenitrile	Nitrile	50.5 (16.8)^a	408.5 (170.9)^b	324.6 (198.8)^{ab}	600.1 (207.3)^b	1219.3 (717.2)^b	**1.17**
5	3-methylbutanenitrile	Nitrile	21.9 (3.6)	56.5 (11.3)	35.7 (12.0)	70.5 (27.2)	227.2 (159.2)	0.82
6	3-methyl-2-pentanone	Ketone	15.7 (3.2)^a	101.3 (35.4)^b	119.3 (35.0)^b	53.1 (9.0)^b	117.7 (36.2)^b	**2.23**
7	2,4-pentanedione	Ketone	16.6 (5.5)	6.5 (1.4)	15.4 (6.1)	5.2 (1.1)	7.5 (2.2)	0.63
8	(*Z*)-3-hexen-1-ol	Alcohol	36.0 (6.6)	136.2 (65.7)	379.3 (157.0)	41.1 (14.7)	275.1 (117.9)	0.58
9	(*Z*)-2-penten-1-ol, acetate	Ester	4.1 (1.6)	17.0 (6.9)	53.9 (20.1)	6.6 (2.7)	16.4 (9.2)	0.64
10	α-thujene	Monoterpene	155.9 (55.6)^a	274.8 (64.5)^{ab}	406.5 (70.7)^b	258.1 (39.9)^{ab}	334.5 (80.5)^{ab}	**1.08**
11	Butylisothiocyanate	Ester	1.0 (0.5)	12.5 (5.5)	8.7 (4.8)	31.6 (14.7)	59.7 (40.1)	0.94
12	α-pinene	Monoterpene	99.4 (19.5)^a	132.2 (21.8)^{ab}	161.5 (22.0)^b	120.3 (13.6)^{ab}	161.6 (30.5)^{ab}	**1.05**
13	sabinene	Monoterpene	28.7 (10.1)^a	51.8 (12.0)^{ab}	75.6 (13.5)^b	47.4 (9.1)^{ab}	59.7 (13.2)^{ab}	**1.07**
14	β-pinene	Monoterpene	8.0 (2.0)^a	12.6 (2.5)^{ab}	17.8 (2.4)^b	12.0 (1.7)^{ab}	16.4 (3.0)^b	**1.45**
15	β-myrcene	Monoterpene	160.0 (49.7)	237.5 (53.1)	333.1 (59.9)	228.8 (34.2)	306.0 (63.1)	**1.24**
16	α-phellandrene	Monoterpene	1.2 (0.4)^a	2.0 (0.5)^{ab}	3.1 (0.7)^b	2.3 (0.6)^{ab}	3.7 (1.4)^{ab}	**1.35**
17	(*Z*)-3-hexen-1-ol, acetate	Ester	372.2 (88.1)	747.2 (330.1)	1933.2 (704.1)	444.9 (167.0)	1172.4 (477.7)	0.60
18	hexyl acetate	Ester	17.6 (5.0)	25.4 (8.9)	92.7 (41.7)	14.4 (3.1)	34.8 (13.3)	**1.11**
19	α-terpinene	Monoterpene	19.1 (7.2)^a	32.1 (8.9)^{ab}	50.6 (12.9)^b	37.3 (9.8)^{ab}	59.9 (24.3)^{ab}	**1.40**
20	1,8-cineole	Monoterpene	38.9 (13.1)^a	67.8 (15.3)^{ab}	93.6 (15.3)^b	64.6 (12.2)^{ab}	83.1 (21.1)^{ab}	**1.47**
21	β-isophorone	Ketone	3.8 (0.8)	6.6 (1.5)	3.4 (0.8)	5.7 (3.1)	4.1 (1.0)	0.36
22	(*E*)-β-ocimene	Monoterpene	6.0 (1.6)	7.2 (1.6)	17.9 (6.3)	6.6 (1.2)	12.4 (3.7)	0.20
23	γ-terpinene	Monoterpene	13.8 (4.2)^a	21.2 (5.0)^{ab}	33.0 (7.8)^b	24.6 (6.3)^{ab}	40.4 (14.6)^{ab}	**1.23**
24	α-terpinolene	Monoterpene	10.6 (2.9)	15.4 (3.6)	22.8 (4.9)	17.2 (3.3)	28.2 (8.7)	0.31

25	Linalool	Monoterpene	7.7 (1.9)	13.2 (6.5)	25.8 (11.3)	5.4 (1.9)	10.4 (3.3)	0.03
26	(*E*)-DMNT	Homoterpene	135.5 (85.2)	126.0 (81.4)	401.3 (178.0)	96.9 (50.9)	104.7 (48.8)	0.71
27	alloocimene	Monoterpene	1.1 (0.3)	1.1 (0.2)	2.1 (0.4)	1.2 (0.3)	1.9 (0.4)	0.70
28	(*Z*)-3-Hexen-1-ol, isobutyrate	Ester	1.4 (0.6)	8.2 (5.1)	63.7 (33.7)	2.4 (0.9)	42.7 (26.4)	0.62
29	1-methyl-4-(1-methylethyl) cyclohexanol	Alcohol	73.2 (49.6)	93.6 (70.5)	144.2 (66.5)	91.3 (56.8)	201.6 (83.4)	0.02
30	α-terpineol	Monoterpene	4.4 (1.8)	6.3 (3.9)	7.2 (1.9)	2.4 (0.4)	4.5 (2.1)	0.14
31	(*Z*)-3-hexenyl isovalerate	Ester	5.1 (1.3)	7.3 (3.6)	58.7 (26.0)	4.8 (1.5)	54.0 (44.2)	0.29
32	verbenone	Monoterpene	9.9 (3.0)	5.0 (1.3)	9.3 (4.4)	4.2 (0.6)	5.5 (0.7)	0.51
33	unknown	NA	27.8 (7.9)	13.1 (1.7)	20.4 (8.8)	11.3 (1.7)	17.1 (2.3)	**1.05**
34	isobornyl acetate	Ester	11.7 (2.2)^{a}	9.1 (2.6)^{ab}	6.8 (2.0)^{b}	9.4 (4.0)^{ab}	8.1 (2.5)^{b}	**1.07**
35	(*Z*)-3-hexen-1-ol, 2-methyl-2-butenoate	Ester	25.6 (4.1)	18.3 (1.9)	36.9 (13.6)	18.3 (3.5)	55.9 (32.7)	0.09
36	unknown	NA	1.3 (0.2)	1.0 (0.1)	1.1 (0.2)	1.0 (0.2)	1.5 (0.2)	0.26
37	isomer of β-elemene	Sesquiterpene	0.3 (0.1)	0.5 (0.4)	1.9 (0.8)	1.2 (0.5)	0.8 (0.5)	0.24
38	β-elemene	Sesquiterpene	4.7 (4.2)	25.2 (18.3)	85.4 (35.8)	61.1 (22.6)	42.1 (25.0)	0.52
39	6,10-dimethyl-2-undecanone	Ketone	21.7 (4.1)	15.2 (2.6)	17.0 (3.5)	16.8 (6.3)	21.9 (3.3)	0.46
40	α-cedrene	Sesquiterpene	6.8 (2.9)	1.2 (0.2)	3.3 (1.4)	1.7 (0.3)	1.4 (0.2)	0.57
41	(*E*)-α-bergamotene	Sesquiterpene	1.2 (0.6)	0.7 (0.5)	2.7 (1.0)	1.9 (0.7)	1.2 (0.7)	0.37
42	(*E*)-β-farnesene	Sesquiterpene	0.3 (0.2)	0.3 (0.1)	1.4 (0.7)	0.6 (0.2)	1.3 (0.9)	0.83
43	β-chamigrene	Sesquiterpene	0.2 (0.1)	0.6 (0.4)	2.3 (1.0)	2.7 (1.2)	1.5 (0.9)	0.65
44	hinesene	Sesquiterpene	0.7 (0.3)^{a}	2.1 (0.9)^{ab}	8.2 (3.0)^{b}	7.5 (3.2)^{ab}	5.2 (2.9)^{ab}	**1.18**
45	α-zingiberene	Sesquiterpene	0.1 (0.1)	2.3 (1.7)	10.3 (4.9)	4.1 (1.5)	3.6 (2.0)	0.99
46	α-selinene	Sesquiterpene	0.7 (0.4)	2.2 (1.5)	10.3 (4.8)	11.8 (5.5)	7.6 (4.8)	0.03
47	cashmeran	Sesquiterpene	5.7 (2.0)^{a}	1.9 (0.2)^{b}	3.1 (1.3)^{ab}	1.9 (0.3)^{b}	2.1 (0.3)^{ab}	**1.29**
48	(*E*,*E*)-α-farnesene	Sesquiterpene	11.3 (4.0)	16.7 (6.0)	52.2 (18.6)	20.2 (7.5)	17.3 (5.0)	0.63
49	β-bisabolene	Sesquiterpene	0.5 (0.2)	2.7 (2.2)	9.1 (4.2)	7.3 (2.8)	4.7 (2.8)	0.21
50	(Z)-γ-bisabolene	Sesquiterpene	0.2 (0.2)	1.2 (0.9)	4.0 (1.7)	2.7 (1.0)	1.8 (1.1)	0.90
	Total volatile emission		29.3 (9.7)^{a}	57.2 (21.5)^{ab}	109.6 (39.1)^{b}	50.8 (15.5)^{ab}	101.0 (44.5)^{b}	

^{x}: Treatments that plants were subjected to: (UD) undamaged control; (PB-S-) ablated *P. brassicae*; (PB-S+) intact *P. brassicae*; (PB-CG-S-) ablated *Cotesia glomerata*-parasitized *P. brassicae*; (PB-CG-S+) intact *C. glomerata*-parasitized *P. brassicae*

Pairwise comparison by PLS-DA for plant volatiles emitted by plants induced by mock-treated and ablated *C. glomerata*-parasitized *P. brassicae* did not result in a significant model when all ten samples for each treatment were included. Using PCA, one outlier sample from mock-treated parasitized caterpillar induced plants was visualized in the score plot (Figure 4c). Upon removing this outlier, subsequent PLS-DA analyses revealed one significant principle component (Figure 4d; PLS-DA, R^2X = 0.256, R^2Y = 0.39, Q^2 = 0.051). In this model, there were 22 compounds with VIP values > 1, including different terpenoids, nitriles, ketones, esters and one alcohol (Figure 4d). Among these compounds, 6,10-dimethyl-2-undecanone and an unknown compound were emitted in higher amounts by plants induced by mock-treated *C. glomerata*-parasitized *P. brassicae* (Mann–Whitney *U* tests, P = 0.049, for both compounds). Moreover, two compounds, namely (*Z*)-3-hexen-1-ol and 1-methyl-4-(1-methylethyl) cyclohexanol, had a marginally significant increase in release by plants induced by mock-treated parasitized caterpillars (Mann–Whitney *U* tests, P = 0.059, for both compounds). In addition, the multivariate analysis did not differentiate volatile blends emitted by plants induced by ablated unparasitized or ablated parasitized *P. brassicae* caterpillars (Figure 4b).

Parasitism induced transcriptional changes in caterpillar labial salivary glands

The *de novo* transcriptome assembly (TA) generated 24,054 contigs (N50 = 2432) that allowed more than 90% of the individual reads used for the combined assembly to be remapped. More than 98% of the total TA-contigs could be remapped with reads corresponding to samples from both caterpillar treatments (Table 2). We identified 7612 sequences (>31%) matching entries in the GenBank nonredundant (NR) database with *E*-value cut-off = 10^{-5}, whereas 16442 sequences (>68%) did not yield matches.

Table 2. Summary statistics for labial salivary glands of *Pieris brassicae* transcriptome sequencing and mapping.

	Salivary Glands - unparasitized Larvae	Salivary Glands - Parasitized Larvae
Total number of reads	158 million	161 million
Read length (bases)	100	100
Reads used for TA-contig assembly	90 million	90 million
Reads used for mapping	145 million	147 million
No. of unmapped reads	9.2 million	10.3 million
No. of TA-contigs not covered by read mappings	353	166

The magnitude of differential transcription in labial salivary glands due to parasitism was visualized by comparing the number of contigs differentially expressed between unparasitized and *C. glomerata* parasitized *P. brassicae* caterpillars (Figure 5). A total of 347 contigs were differentially expressed in labial salivary glands between

unparasitized and parasitized caterpillars (false discovery rate, P < 0.05; fold change > 2). There were 237 contigs with higher expression in salivary glands extracted from parasitized caterpillars, whereas 110 contigs were expressed more strongly in salivary glands of unparasitized caterpillars (Table S1).

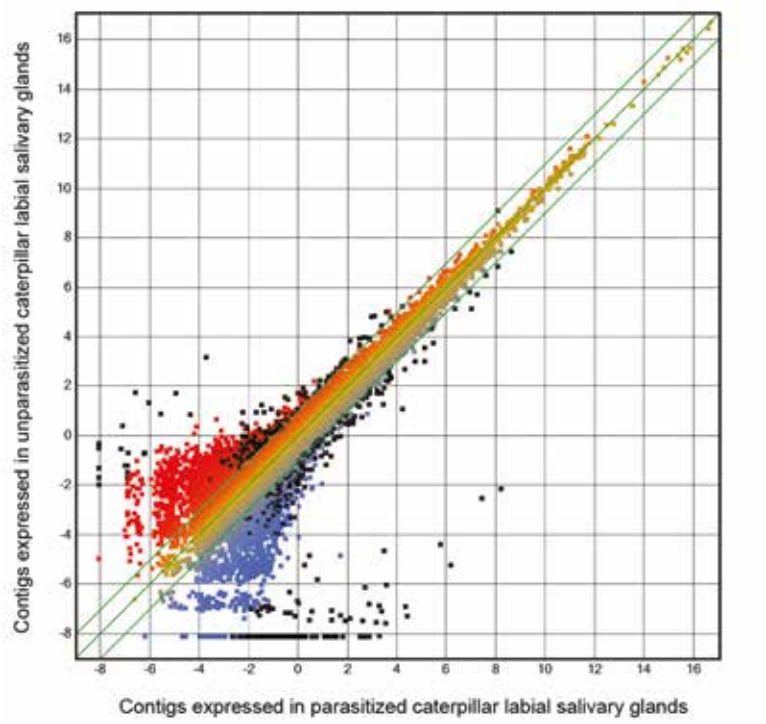

Figure 5. Scatter plot showing global gene expression in labial salivary glands of *Pieris brassicae* isolated from unparasitized (Y-axis) or *Cotesia glomerata* parasitized (X-axis) caterpillars. Shown are log2 transformed RPKM values. Colour indicates expression ratios of contigs that fall within a 2-fold cutoff. Contigs with expression ratios greater than 2-fold are shown in red (associated with labial salivary glands of unparasitized *P. brassicae*) or in blue (associated with labial salivary glands of parasitized *P. brassicae*). Contigs with expression ratios greater than 2-fold and P < 0.05 (FDA) are shown in black.

Gene ontology-enrichment analysis revealed that nutrient reservoir activity was overrepresented in salivary glands of unparasitized caterpillars (Figure 6). In contrast, the GO terms that were over-represented in salivary glands of *C. glomerata* parasitized caterpillars included modulation of host processes by viruses and virus suppression of host NF-kappa B transcription factor (Figure 6). Interestingly, we found that the expression of genes encoding β-glucosidase as well as storage proteins involved in growth and development were suppressed in salivary glands of parasitized caterpillars (Table S1). Some other proteins with suppression in salivary glands of parasitized caterpillars were cuticle proteins, e3 ubiquitin-protein ligase, distal antenna-like protein,

and latrophilin-like receptor (Table S1). In contrast, glucose oxidase (GOX), an enzyme contributing to suppression of plant defences, was up-regulated in salivary glands of parasitized caterpillars (Table S1). Some other genes up-regulated in salivary glands of parasitized caterpillars were those that code for Krueppel homologs, arylsulfatase B, trehalase and trehalose transporters, and β-fructofuranosidase (Table S1).

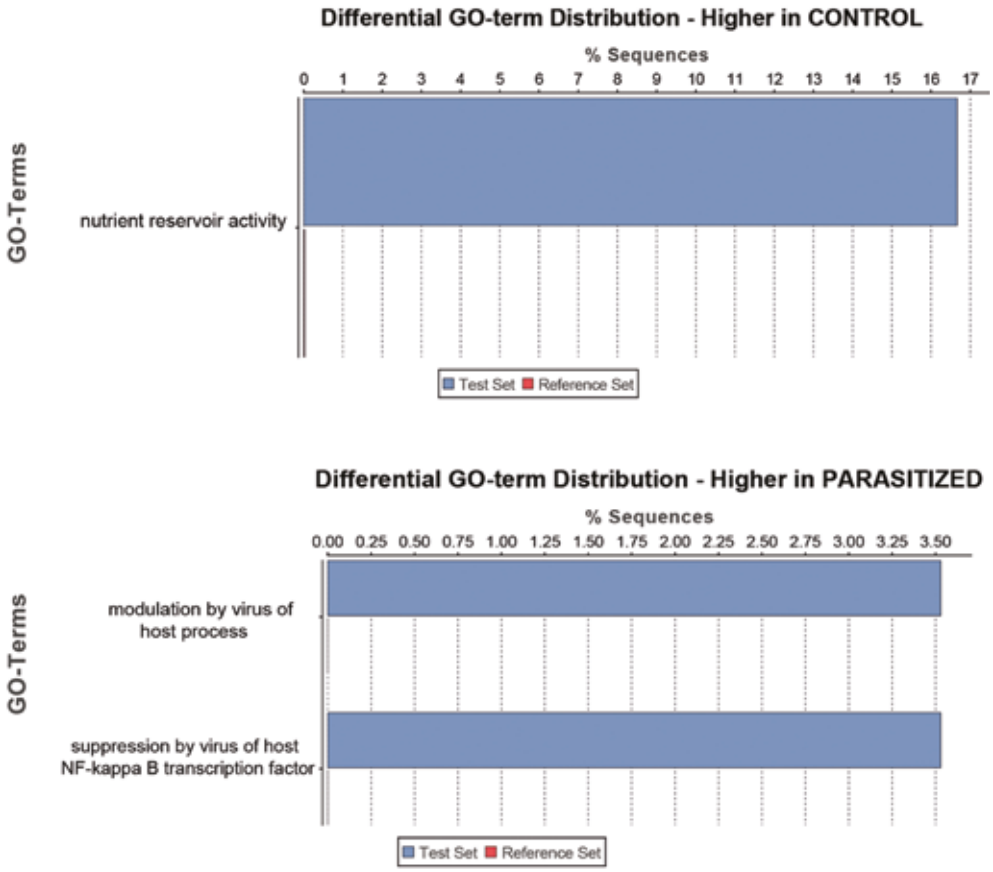

Figure 6. GO-enrichment analysis for contigs with up-regulation in labial salivary glands of either unparasitized *Pieris brassicae* caterpillar (upper panel) or *Cotesia glomerata* parasitized caterpillars (lower panel).

Discussion

In the current study, we demonstrated an altered herbivore-induced plant trait-mediated indirect interaction network by eliminating caterpillar saliva secretion using an ablation technique for labial salivary glands. Our previous studies had revealed that the hyperparasitoid *L. nana* exploits HIPVs as cues for host searching and that

plants damaged by parasitized caterpillars were more attractive to *L. nana* than plants damaged by healthy caterpillars (Poelman *et al.* 2012; Zhu *et al.* 2015). However, when the labial salivary glands of the caterpillars were completely removed, plants induced by either unparasitized or parasitized caterpillars showed equal attractiveness to *L. nana* and plants became less attractive to *L. nana* when damaged by ablated caterpillars compared to plants damaged by mock-treated caterpillars (Figure 1b). Furthermore *L. nana* were not able to distinguish herbivore-damaged plants from undamaged control plants when caterpillar salivary glands had been removed (Figure 1b). Similarly, we found that plant phenotypes induced by ablated caterpillars had strong effects on oviposition preference of *P. xylostella* moths, eliminating the previously observed preference for plants damaged by unparasitized over plants damaged by parasitized caterpillars (Poelman *et al.* 2011b) as well as the preference for damaged over undamaged plants (Poelman *et al.* 2008a; Bruinsma *et al.* 2010). Therefore, these results indicate that caterpillar labial saliva plays a crucial role in plant-herbivore interactions by affecting the plant phenotype, and thereby affecting plant-mediated multitrophic interactions. When labial saliva secretion had been abolished, plants lost part of their induced phenotype in response to herbivory, and may perceive herbivory similar to mechanical damage (Mithofer *et al.* 2005; Bricchi *et al.* 2010). For the hyperparasitoid, the interaction of labial saliva of the host herbivore that contains the parasitoid larvae's signature with the plant allows them to locate their hosts. *Plutella xylostella* does distinguish the different plant phenotypes induced by intact unparasitized or parasitized caterpillars. However, when salivary glands had been ablated in both types of caterpillars (healthy and parasitized), the effect of herbivory on trait-mediated species interactions with the moth and the hyperparasitoid were lost. This further indicates that parasitism causes physiological changes in caterpillar labial salivary glands, which elicit different plant responses to damage by unparasitized or parasitized caterpillars.

To further investigate the tissue-specific differences in caterpillar labial salivary glands of healthy caterpillars and *C. glomerata* parasitized caterpillars, we carried out transcriptome sequencing. Our results revealed a clear transcriptional difference in salivary glands isolated from unparasitized or parasitized caterpillars. Interestingly, we found transcripts of two important herbivore-associated elicitors in the labial salivary glands of *P. brassicae* to be affected by parasitism, namely β-glucosidase and glucose oxidase (GOX). In plants, β-glucosidases are involved in bio-activation of major defensive secondary metabolites, such as cyanogenic glucosides, benzoxazinoid glucosides, avenacosides and glucosinolates (Morant *et al.* 2008). β-Glucosidase was identified in regurgitant of *P. brassicae* and is able to induce a plant volatile blend comparable to HIPVs induced by actual *P. brassicae* feeding, resulting in the attraction of specialist parasitoids of the herbivore (Mattiacci *et al.* 1995). In addition,

the enzyme GOX interferes with the activation of plant defence responses and has been identified in different Lepidopteran species (Eichenseer *et al.* 2010; Bonaventure 2012). Previous studies revealed that GOX in *Helicoverpa zea* saliva contributes to suppression of the defence responses of *Nicotiana tabacum* plants (Musser *et al.* 2002), whereas GOX elicits defence responses in *Solanum lycopersicum* plants (Tian *et al.* 2012). In *brassica*ceous plants, GOX suppresses the expression of wound-induced genes in *Arabidopsis thaliana* (Consales *et al.* 2012). Our transcriptome sequencing results show a suppressed expression of β-glucosidase in salivary glands of parasitized *P. brassicae* caterpillars, in contrast an upregulated expression of genes encoding GOX. Therefore, this indicates that the presence of parasitoid larvae affects the expression of herbivore-associated elicitors in caterpillar labial saliva, suppressing a positive regulator and inducing a negative regulator of plant defensive responses to herbivory. The performance of parasitoids may be negatively affected when the herbivorous host feeds on a chemically defended plant (Fortuna *et al.* 2014). Moreover, the performance of a parasitoid and its herbivore host are often positively correlated (Bukovinszky *et al.* 2009; Gols & Harvey 2009). From the parasitoid's point of view, they may benefit from manipulating the elicitors in the saliva of the host herbivore in order to suppress defence of the host plant. Nevertheless, the presence of parasitoid larvae indirectly interferes with plant responses to herbivory, whereas these altered plant phenotypes allow *P. xylostella* moths and hyperparasitoids to discriminate plants infested by unparasitized or parasitized herbivores such that the parasitoids will experience herbivore-mediated competition as well as risks of being parasitized by hyperparasitoids.

Upon herbivore attack, plants release HIPVs that attract natural enemies of the herbivore as "bodyguards" (Takabayashi & Dicke 1996). As a public source of information, HIPVs are not only perceived by natural enemies of herbivores, but also mediate a wide range of interactions among other community members, including hyperparasitoids (Dicke & Baldwin 2010; Poelman *et al.* 2012). To establish plant-volatile-mediated interactions between plants and hyperparasitoids, components in herbivore oral secretion are required as mechanical damage only is not sufficient (Poelman *et al.* 2012). Here, total emission rate of plant volatiles induced by ablated caterpillars was similar to the emission rate of undamaged control plants, which suggests that caterpillar labial saliva is required for the induction of a plant response to herbivory (Bricchi *et al.* 2010). Plant volatiles induced by ablated caterpillars did not differ qualitatively from volatiles emitted by plants induced by mock-treated caterpillars, but they did differ quantitatively (Figure 2). Compared to plant volatiles induced by mock-treated parasitized caterpillars, the green-leaf volatile (*Z*)-3-hexen-1-ol was emitted in relatively lower amount by plants induced by ablated parasitized caterpillars. Moreover, two typical HIPVs, (*E*)-DMNT and (*E*,*E*)-α-farnesene (Arimura

et al. 2004; Mumm & Dicke 2010; Weldegergis *et al.* 2015), showed a significantly reduced emission from plants damaged by ablated unparasitized caterpillars compared to mock-treated unparasitized caterpillar-induced plants. (*E*)-DMNT has been detected in cultivated *B. oleracea* plants damaged by parasitized *Pieris* caterpillars and was suggested to be involved in plant-hyperparasitoid interactions (Poelman *et al.* 2012).

In the present study, we show that parasitism by the endoparasitoid *C. glomerata* alters the composition of *P. brassicae* labial saliva including effects on the transcription of genes coding for well-known herbivore-associated elicitors, β-glucosidase and GOX, and greatly contributes to herbivore-induced plant trait-mediated indirect interaction networks. Although plants infested with healthy herbivores or parasitized herbivores differentially mediate indirect interaction networks, the differences were lost when the key eliciting factor, labial saliva, was eliminated. Our study further contributes to a better understanding of the key elements that regulate plant trait-mediated indirect interactions, showing that parasitoids feeding in the herbivores are a significant force affecting this multitrophic interaction network via manipulation of the physiology of the herbivore host (Kaplan 2012). The parasitoids live in the haemocoel of their host. Future studies should elucidate the mechanisms through which the haemocoel-located parasitoid larvae manipulate transcriptional responses of the host's salivary glands.

Our work highlights the intricate way in which species can modulate indirect species interactions: parasitoid larvae that do not contact the plant influence the plant phenotype by influencing the phenotype of their herbivorous host. Taking this knowledge of the mechanisms underlying indirect species interactions to the field to investigate the consequences for the wider plant-associated community will be an important next step that will aid in understanding the dynamics of such communities.

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Supporting information

Table S1. Contigs with expression ratios greater than 2-fold and P < 0.05 cutoffs in labial salivary glands of unparasitized (PB) or *Cotesia glomerata* parasitized (PB-CG) *Pieris brassicae.*

Name	Seq. Length	Seq. Description	Fold change (PB-CG vs. PB)	P-value
ASS2_C6243	807	hypothetical protein BV9-4	434.046 up	3.49E-08
ASS2_C661	570	bv9 family protein	2713.858 up	3.81E-08
ASS2_C11309	267	---NA---	1697.054 up	3.81E-08
ASS2_C19060	393	---NA---	826.225 up	3.81E-08
ASS2_C7293	495	viral ankyrin	396.114 up	3.81E-08
ASS2_C10750	325	ben domain protein	618.406 up	3.81E-08
ASS2_C17272	736	---NA---	228.266 up	3.81E-08
ASS2_C8771	328	conserved hypothetical protein	595.516 up	3.81E-08
ASS2_C7728	1444	bv6 family protein	2018.533 up	3.81E-08
ASS2_C12266	765	bv21 family protein	465.666 up	3.86E-08
ASS2_C6996	1671	ben domain protein	1576.456 up	4.00E-08
ASS2_C11725	592	host translation inhibitory factor ii	968.504 up	4.00E-08
ASS2_C7673	427	hypothetical protein CcBV_3.3	781.753 up	5.37E-08
ASS2_C14669	272	hypothetical protein BV19-1	248.446 up	7.27E-08
ASS2_C16007	401	viral ankyrin	185.324 up	7.90E-08
ASS2_C8772	377	conserved hypothetical protein	1825.395 up	1.02E-07
ASS2_C1195	938	bv8 family protein	992.447 up	2.15E-07
ASS2_C7326	441	---NA---	278.258 up	4.00E-07
ASS2_C6451	1020	---NA---	572.207 up	4.03E-07
ASS2_C15237	469	---NA---	700.588 up	4.29E-07
ASS2_C22167	240	---NA---	114.425 up	4.87E-07
ASS2_C15618	391	conserved hypothetical ben domain protein	222.547 up	5.31E-07
ASS2_C13627	653	---NA---	333.185 up	5.31E-07
ASS2_C18005	305	elongation factor 1-alpha 1	168.449 up	5.81E-07
ASS2_C18324	476	conserved hypothetical protein	330.481 up	5.81E-07
ASS2_C10675	751	bv6 family protein	573.821 up	6.96E-07
ASS2_C18414	568	ben domain protein	180.534 up	8.25E-07
ASS2_C18393	304	60s ribosomal protein l18	90.335 up	8.77E-07
ASS2_C10616	325	---NA---	339.431 up	1.27E-06
ASS2_C16839	609	---NA---	209.063 up	1.33E-06
ASS2_C19247	330	40s ribosomal protein s3a	163.773 up	1.35E-06
ASS2_C15189	801	serine proteinase stubble-like	291.707 up	1.35E-06
ASS2_C1718	3268	ben domain protein	2213.795 up	1.36E-06
ASS2_C14161	322	---NA---	122.092 up	1.36E-06
ASS2_C21768	222	---NA---	190.295 up	1.48E-06
ASS2_C21673	408	arylphorin subunit alpha	140.987 up	1.57E-06
ASS2_C19831	243	elongation factor 1 partial	227.771 up	1.73E-06
ASS2_C14624	284	elongation factor 1- partial	258.977 up	1.82E-06

ASS2_C14301	317	conserved hypothetical ben domain protein	169.293 up	3.06E-06
ASS2_C21746	253	protein disulfide-isomerase a6	145.178 up	3.42E-06
ASS2_C18018	356	protein disulfide-isomerase a3	136.833 up	3.53E-06
ASS2_C23282	296	hexamerin	136.362 up	4.15E-06
ASS2_C16515	288	---NA---	111.581 up	4.38E-06
ASS2_C17856	763	arylphorin subunit alpha	188.305 up	4.41E-06
ASS2_C18848	213	conserved hypothetical ben domain protein	164.110 up	4.99E-06
ASS2_C13830	273	conserved hypothetical ben domain protein	205.254 up	5.20E-06
ASS2_C5956	205	---NA---	1686.710 up	7.27E-06
ASS2_C15682	521	heat shock 70 kda protein cognate 3	291.209 up	7.27E-06
ASS2_C7462	622	ben domain protein	190.488 up	8.85E-06
ASS2_C18758	270	atp-dependent rna helicase	456.282 up	9.28E-06
ASS2_C22276	303	---NA---	72.078 up	1.01E-05
ASS2_C21636	281	beta-glucosidase precursor	117.647 up	1.04E-05
ASS2_C22308	234	ribosomal protein l21	91.838 up	1.36E-05
ASS2_C555	825	hypothetical protein CcBV_26.4	1286.045 up	1.56E-05
ASS2_C4762	243	---NA---	159.196 up	1.86E-05
ASS2_C13012	540	---NA---	247.138 up	1.86E-05
ASS2_C12220	463	---NA---	609.757 up	1.92E-05
ASS2_C22211	261	---NA---	155.129 up	2.09E-05
ASS2_C12750	524	conserved hypothetical ben domain protein	150.053 up	2.22E-05
ASS2_C14901	339	hypothetical protein 32.18	96.336 up	2.47E-05
ASS2_C17042	284	conserved hypothetical ben domain protein	81.312 up	2.97E-05
ASS2_C13786	221	---NA---	200.294 up	3.28E-05
ASS2_C4390	964	ben domain protein	274.449 up	4.12E-05
ASS2_C19222	229	---NA---	113.415 up	4.12E-05
ASS2_C17335	246	---NA---	149.309 up	4.12E-05
ASS2_C12779	287	dihydrolipoyllysine-residue acetyltransferase component 2 of pyruvate dehydrogenase mitochondrial isoform x1	145.864 up	4.15E-05
ASS2_C12510	312	hexamerin	235.745 up	4.25E-05
ASS2_C12927	823	protein disulfide-isomerase a6	168.380 up	4.61E-05
ASS2_C17285	244	---NA---	203.201 up	4.61E-05
ASS2_C20939	276	---NA---	87.571 up	4.61E-05
ASS2_C23568	426	arylphorin subunit alpha	163.075 up	4.71E-05
ASS2_C15242	402	conserved hypothetical ben domain protein	154.628 up	4.71E-05
ASS2_C11672	401	ep1-like protein	152.750 up	4.97E-05
ASS2_C16786	324	ben domain protein	117.949 up	5.33E-05
ASS2_C13301	291	---NA---	160.661 up	8.24E-05
ASS2_C9775	1201	ben domain protein	1622.121 up	8.45E-05
ASS2_C21223	220	---NA---	59.027 up	8.94E-05
ASS2_C15856	520	protein npc2 homolog	137.412 up	9.42E-05
ASS2_C14303	437	ben domain protein	102.217 up	0.000104
ASS2_C10688	311	---NA---	116.212 up	0.000133

ASS2_C14871	1304	bv21 family protein	37.601 up	0.000148
ASS2_C4186	3227	melanization-related protein	1075.936 up	0.000165
ASS2_C2834	2020	arylsulfatase b	8.706 up	0.000175
ASS2_C17017	409	---NA---	97.246 up	0.000198
ASS2_C21156	327	hexamerin-like	94.453 up	0.000202
ASS2_C9953	344	hypothetical protein BV22-2	100.131 up	0.000227
ASS2_C20401	391	protein npc2 homolog	194.104 up	0.000263
ASS2_C6063	1052	protein tyrosine phosphatase	650.031 up	0.00031
ASS2_C7025	646	bv6 family protein	3322.083 up	0.000317
ASS2_C17723	232	aminopeptidase n	73.666 up	0.000366
ASS2_C5385	1275	bv8 family protein	1123.172 up	0.000429
ASS2_C12039	649	hypothetical protein CcBV_19.4	150.399 up	0.000472
ASS2_C16807	344	---NA---	86.837 up	0.000474
ASS2_C17992	396	serine carboxypeptidase precursor family protein	75.434 up	0.00053
ASS2_C9212	838	---NA---	708.745 up	0.00056
ASS2_C11871	467	transmembrane and tpr repeat-containing protein 1-like	7.996 up	0.000691
ASS2_C23037	419	histone h2b	116.479 up	0.000713
ASS2_C22179	275	---NA---	65.713 up	0.000774
ASS2_C5135	3044	rna-directed dna polymerase from mobile element jockey-like	813.902 up	0.000787
ASS2_C17404	268	---NA---	2.531 up	0.000795
ASS2_C12820	967	ser-rich protein	435.474 up	0.000797
ASS2_C906	2344	ben domain protein	769.345 up	0.000804
ASS2_C4656	785	leucine-rich repeat-containing protein ddb_g0290503-like	4.397 up	0.000804
ASS2_C19360	241	hypothetical protein CAPTEDRAFT_206368	113.950 up	0.000864
ASS2_C19170	237	conserved hypothetical protein	97.438 up	0.00095
ASS2_C1396	1784	cytochrome p450	3.009 up	0.000961
ASS2_C2927	1266	viral ankyrin	2703.799 up	0.00102
ASS2_C21731	274	60s ribosomal protein l5	74.936 up	0.00102
ASS2_C10328	1016	calreticulin	240.053 up	0.00107
ASS2_C2748	2973	glucose dehydrogenase	2.451 up	0.00115
ASS2_C18686	248	---NA---	97.458 up	0.00122
ASS2_C2579	1545	alpha-tocopherol transfer	2.811 up	0.00128
ASS2_C16634	460	---NA---	8.862 up	0.00129
ASS2_C22056	237	ep1-like protein	169.234 up	0.00159
ASS2_C21726	263	---NA---	72.572 up	0.00177
ASS2_C4389	2901	ben domain protein	492.251 up	0.00179
ASS2_C17835	259	coatomer subunit partial	68.579 up	0.00182
ASS2_C12167	650	neutral endopeptidase	4.225 up	0.00212
ASS2_C6402	825	---NA---	1393.452 up	0.00224
ASS2_C3259	1718	aromatic-l-amino-acid decarboxylase-like	3.517 up	0.00252
ASS2_C16516	339	60s ribosomal protein l18a	65.883 up	0.0027
ASS2_C6329	1107	hydroxybutyrate dehydrogenase	2.645 up	0.0027

ASS2_C19889	241	---NA---	43.291 up	0.00318
ASS2_C9865	1685	---NA---	3.393 up	0.00324
ASS2_C18866	279	hypothetical protein KGM_00511	46.544 up	0.00347
ASS2_C5370	211	---NA---	2371.357 up	0.00377
ASS2_C3834	2228	nucleolar complex protein 2 homolog	2.196 up	0.00397
ASS2_C19955	290	---NA---	3.617 up	0.0042
ASS2_C15755	350	ben domain protein	73.914 up	0.0046
ASS2_C4199	204	---NA---	4.251 up	0.00485
ASS2_C22720	325	hypotetical protein bv4-1	67.888 up	0.00497
ASS2_C6596	2538	facilitated trehalose transporter tret1-like	2.878 up	0.00581
ASS2_C5206	3998	thrombospondin type-1 domain-containing protein 7a	2.028 up	0.00602
ASS2_C16852	276	histone h4	81.869 up	0.00606
ASS2_C6876	3280	2-oxoglutarate dehydrogenase	2.317 up	0.00644
ASS2_C10997	531	von willebrand factor d and egf domain-containing protein	3.282 up	0.00644
ASS2_C13051	201	---NA---	1149.161 up	0.00672
ASS2_C553	1349	heat shock 70 kda protein cognate 3 isoform x1	108.024 up	0.00681
ASS2_C9660	659	bv9 family protein	142.869 up	0.00735
ASS2_C3009	374	cg10200	3.222 up	0.00742
ASS2_C18001	355	---NA---	123.121 up	0.00787
ASS2_C14542	356	ubiquitin-activating enzyme e1	67.892 up	0.00828
ASS2_C6446	1390	facilitated trehalose transporter tret1-like	3.362 up	0.00828
ASS2_C16830	512	retrovirus-related pol polyprotein from transposon 412	7.828 up	0.00828
ASS2_C6652	651	apolipoprotein d-like isoform x2	337.219 up	0.00842
ASS2_C9184	3112	disintegrin and metalloproteinase domain-containing protein 12-like	3.399 up	0.00881
ASS2_C2404	750	ben domain protein	448.924 up	0.0101
ASS2_C14310	858	---NA---	2.569 up	0.0106
ASS2_C19055	293	cytochrome p450	5.070 up	0.0107
ASS2_C4550	357	ornithine decarboxylase	2.966 up	0.0111
ASS2_C262	2528	heat shock protein 90	2.201 up	0.0111
ASS2_C6635	1135	ben domain protein	95.765 up	0.0117
ASS2_C12660	708	ecdysone-inducible protein partial	4.549 up	0.0117
ASS2_C17829	1283	ovalbumin-related protein x isoform x12	98.484 up	0.0117
ASS2_C12102	789	ben domain protein	191.869 up	0.0128
ASS2_C19713	388	neurotransmitter gated ion channel	7.291 up	0.0132
ASS2_C10039	330	---NA---	2.728 up	0.0133
ASS2_C11872	821	beta lysosomal	3.660 up	0.0133
ASS2_C9654	757	lysozyme-like	2.148 up	0.0136
ASS2_C1949	1619	sucrose-6-phosphate hydrolase	2.141 up	0.0138
ASS2_C890	568	cuticle protein cpg43	2.042 up	0.014
ASS2_C18418	295	---NA---	2.477 up	0.015
ASS2_C12797	1135	glycerophosphoryl diester periplasmic	3.883 up	0.015

ASS2_C9803	207	---NA---	2.821 up	0.0154
ASS2_C3326	1332	apolipoprotein d	2.694 up	0.0154
ASS2_C14371	473	ben domain protein	98.277 up	0.0159
ASS2_C10930	1276	hypothetical protein KGM_08735	2.209 up	0.0161
ASS2_C12603	780	atp synthase subunit mitochondrial-like	5.542 up	0.0162
ASS2_C9754	1738	cysteine synthase	2.053 up	0.0168
ASS2_C4142	245	---NA---	3.002 up	0.0169
ASS2_C12843	265	hypothetical protein KGM_04641	2.355 up	0.0174
ASS2_C11363	456	---NA---	7.800 up	0.0176
ASS2_C14682	1812	mind- isoform b	4.189 up	0.0176
ASS2_C17144	407	---NA---	110.267 up	0.0195
ASS2_C7818	1085	arylalkylamine n-acetyltransferase	2.983 up	0.0195
ASS2_C2740	316	alpha amylase	2.445 up	0.0199
ASS2_C14422	388	---NA---	2.111 up	0.0202
ASS2_C15995	471	---NA---	3.655 up	0.0203
ASS2_C15455	284	---NA---	2.800 up	0.0212
ASS2_C6991	1437	glycine n-methyltransferase-like	2.609 up	0.0213
ASS2_C8823	378	---NA---	3.199 up	0.0221
ASS2_C9164	943	inosine-uridine preferring nucleoside hydrolase	3.187 up	0.0222
ASS2_C6324	1165	aldose 1-epimerase	2.269 up	0.0234
ASS2_C4454	892	hypothetical protein KGM_07240	2.716 up	0.0248
ASS2_C22586	360	cytosolic carboxypeptidase -like	5.212 up	0.0257
ASS2_C20233	599	---NA---	50.638 up	0.0259
ASS2_C7559	1656	organic cation transporter	2.892 up	0.026
ASS2_C7800	462	igf2 mrna binding protein	2.131 up	0.026
ASS2_C20012	423	aldehyde dehydrogenase family 1 member l1-like isoform 1	2.802 up	0.0261
ASS2_C23238	395	elongation of very long chain fatty acids protein 4	54.100 up	0.0264
ASS2_C18901	378	---NA---	2.416 up	0.0266
ASS2_C3051	679	---NA---	2.817 up	0.0271
ASS2_C6162	268	---NA---	2.257 up	0.0272
ASS2_C5771	255	---NA---	3.147 up	0.0273
ASS2_C5644	1122	calcitonin receptor	2.047 up	0.0275
ASS2_C18143	247	---NA---	2.645 up	0.0278
ASS2_C9198	883	elongation of very long chain fatty acids protein 4	2.110 up	0.0286
ASS2_C20174	475	PREDICTED: uncharacterized protein LOC101736715	4.707 up	0.0294
ASS2_C674	1483	neurofilament heavy polypeptide-like isoform x2	2.049 up	0.0297
ASS2_C20479	276	zinc finger protein 177-like	2.375 up	0.0303
ASS2_C11207	246	---NA---	3.145 up	0.0303
ASS2_C1153	2228	nucleolar protein 66	2.315 up	0.0305
ASS2_C8534	447	armadillo repeat-containing protein 3-like	3.473 up	0.0308
ASS2_C18280	639	membrane metallo-endopeptidase-like 1-like	2.384 up	0.0314
ASS2_C10540	586	hypothetical protein CcBV_28.4	63.045 up	0.0321

ASS2_C14407	651	isoform c	2.444 up	0.034
ASS2_C18749	560	organic cation transporter	4.761 up	0.0342
ASS2_C16763	274	---NA---	2.460 up	0.0346
ASS2_C16235	344	transcription factor e75a	3.247 up	0.0356
ASS2_C18400	520	isoform f	3.404 up	0.0361
ASS2_C8032	329	hypothetical protein KGM_17951	2.743 up	0.0382
ASS2_C6744	530	cg10035-pa	4.866 up	0.0382
ASS2_C14585	390	cytoplasmic polyadenylation element-binding protein 1-like	4.233 up	0.0385
ASS2_C20382	291	bv6 family protein	62.058 up	0.039
ASS2_C8861	256	---NA---	2.049 up	0.0391
ASS2_C8860	1655	kruppel homolog 1	43.687 up	0.0417
ASS2_C14356	941	zinc finger protein	2.000 up	0.0423
ASS2_C8363	283	---NA---	2.484 up	0.0431
ASS2_C18313	438	hypothetical protein TcasGA2_TC002700	42.344 up	0.0431
ASS2_C15012	623	btb poz domain-containing protein kctd1-like	4.273 up	0.0433
ASS2_C18584	563	---NA---	2.457 up	0.0433
ASS2_C18556	532	isoform c	2.585 up	0.0446
ASS2_C10040	872	sarcoplasmic calcium-binding	2.765 up	0.0452
ASS2_C21175	396	transmembrane and tpr repeat-containing protein 1-like	2.311 up	0.0456
ASS2_C16709	386	---NA---	3.393 up	0.0462
ASS2_C10828	774	PREDICTED: uncharacterized protein LOC101741240	3.138 up	0.0473
ASS2_C4132	542	acyl- z9 desaturase	3.030 up	0.0473
ASS2_C17441	317	---NA---	5.155 up	0.0475
ASS2_C14433	1052	cuticular protein analogous to peritrophins 1-g	2.034 up	0.0475
ASS2_C1185	1146	PREDICTED: uncharacterized protein LOC101741030	2.321 up	0.0476
ASS2_C17735	467	isoform a	3.955 up	0.0481
ASS2_C4686	1624	venom acid phosphatase acph-1-like	2.183 up	0.0482
ASS2_C11385	209	---NA---	2.101 up	0.0482
ASS2_C13136	622	pdz and lim domain protein 3-like	3.187 up	0.0488
ASS2_C3752	1388	trehalase- partial	8.482 up	0.0491
ASS2_C17278	595	---NA---	3.195 up	0.0492
ASS2_C6256	1506	leucine zipper tumor suppressor 2 homolog	2.076 up	0.0492
ASS2_C22389	402	---NA---	2.171 down	0.05
ASS2_C15400	329	---NA---	3.548 down	0.0493
ASS2_C23944	326	takeout jhbp like protein	57.415 down	0.0492
ASS2_C15079	486	---NA---	2.259 down	0.0492
ASS2_C16598	418	---NA---	2.236 down	0.0484
ASS2_C20434	434	isoform c	5.047 down	0.0483
ASS2_C17284	393	---NA---	2.254 down	0.0482
ASS2_C20893	264	calbindin-32 isoform x2	2.777 down	0.0475
ASS2_C13815	775	integrase core domain protein	2.079 down	0.0469

ASS2_C13756	637	monocarboxylate transporter	3.344 down	0.0469
ASS2_C22905	344	interferon gamma induced gtpase	4.192 down	0.0466
ASS2_C16876	665	---NA---	2.131 down	0.0443
ASS2_C14032	292	PREDICTED: uncharacterized protein LOC101743931	2.028 down	0.0443
ASS2_C21068	417	endonuclease and reverse transcriptase-like protein	2.188 down	0.0443
ASS2_C18160	314	---NA---	2.121 down	0.0441
ASS2_C13537	857	latrophilin-like receptor	2.308 down	0.0436
ASS2_C16866	554	---NA---	3.046 down	0.0436
ASS2_C15978	528	---NA---	2.502 down	0.0436
ASS2_C23861	239	non-ltr retrotransposon cats	75.556 down	0.0435
ASS2_C21827	283	---NA---	2.955 down	0.0431
ASS2_C7737	799	---NA---	2.152 down	0.0431
ASS2_C13465	773	---NA---	2.499 down	0.0431
ASS2_C18815	406	---NA---	2.422 down	0.0423
ASS2_C17655	329	---NA---	2.878 down	0.0423
ASS2_C23434	275	---NA---	5.704 down	0.0415
ASS2_C15835	427	---NA---	2.344 down	0.0412
ASS2_C9788	1184	---NA---	2.038 down	0.0404
ASS2_C19374	337	eukaryotic peptide chain release factor subunit 1-like isoform	2.256 down	0.0401
ASS2_C21090	456	---NA---	2.096 down	0.04
ASS2_C16061	559	---NA---	2.152 down	0.0398
ASS2_C10171	1776	hypothetical protein KGM_22069	2.657 down	0.0386
ASS2_C17795	348	---NA---	3.447 down	0.0385
ASS2_C10175	295	---NA---	2.420 down	0.0378
ASS2_C22330	359	---NA---	2.059 down	0.0369
ASS2_C3899	637	uncharacterized atp-dependent helicase yhr031c	2.576 down	0.0368
ASS2_C20792	520	larval cuticle protein lcp-17-like	9.364 down	0.0363
ASS2_C15062	682	---NA---	7.235 down	0.0358
ASS2_C15285	383	---NA---	2.378 down	0.0356
ASS2_C20078	559	heat shock protein	2.739 down	0.0356
ASS2_C7679	1639	reverse transcriptase	2.393 down	0.0356
ASS2_C1968	1866	repeat element protein-	2.293 down	0.0354
ASS2_C9689	432	---NA---	2.176 down	0.0345
ASS2_C19022	524	---NA---	2.816 down	0.0344
ASS2_C12420	306	---NA---	2.927 down	0.0342
ASS2_C15150	1048	---NA---	2.052 down	0.0337
ASS2_C12333	829	---NA---	2.536 down	0.0327
ASS2_C18472	1701	nephrin isoform x1	6.635 down	0.0304
ASS2_C15308	1080	hypothetical protein KGM_00708	2.753 down	0.0304
ASS2_C16540	456	hypothetical protein KGM_10651	3.111 down	0.0299
ASS2_C16106	657	---NA---	2.577 down	0.0299
ASS2_C13987	827	prophenoloxidase subunit 1	2.057 down	0.0295

ASS2_C18595	404	orphan nuclear receptor e75c	6.300 down	0.0291
ASS2_C6557	662	---NA---	2.042 down	0.0271
ASS2_C22571	242	zinc finger protein 271 (zinc finger protein 7) (zinc finger protein znfphex133) (epstein-barr virus-induced zinc finger protein) (znf-eb) (ct-zfp48) (zinc finger protein	4.583 down	0.0271
ASS2_C17270	1058	---NA---	3.063 down	0.026
ASS2_C6089	394	---NA---	2.067 down	0.0257
ASS2_C12619	389	---NA---	2.839 down	0.0256
ASS2_C13101	1488	protein takeout-like	3.745 down	0.0248
ASS2_C9311	750	PREDICTED: uncharacterized protein LOC101746304	4.712 down	0.0243
ASS2_C21973	497	storage protein 1	98.721 down	0.0243
ASS2_C12643	782	polypeptide n-acetylgalactosaminyltransferase 9-like isoform	2.277 down	0.0237
ASS2_C19871	374	mutant cadherin	3.622 down	0.0234
ASS2_C17798	447	---NA---	3.323 down	0.0227
ASS2_C11419	241	---NA---	2.086 down	0.0222
ASS2_C9045	507	wd repeat-containing protein 81	2.006 down	0.0222
ASS2_C20089	519	---NA---	2.188 down	0.0212
ASS2_C15414	1427	---NA---	2.024 down	0.0211
ASS2_C17473	812	---NA---	2.843 down	0.0203
ASS2_C10757	845	protein cubitus interruptus	2.397 down	0.0202
ASS2_C20296	456	---NA---	2.515 down	0.0199
ASS2_C17003	412	nesprin-1-like isoform x2	2.209 down	0.0195
ASS2_C11371	1150	---NA---	2.409 down	0.0187
ASS2_C9753	831	---NA---	2.423 down	0.0185
ASS2_C17413	664	nascent polypeptide-associated complex subunit muscle-specific form-like	2.187 down	0.0176
ASS2_C13534	488	---NA---	3.955 down	0.017
ASS2_C875	521	---NA---	2.803 down	0.0154
ASS2_C23936	472	cuticular protein rr-1 motif 46	190.735 down	0.015
ASS2_C20818	2329	moderately methionine rich storage protein	333.589 down	0.014
ASS2_C4875	2749	PREDICTED: uncharacterized protein LOC763787	3.495 down	0.014
ASS2_C5226	322	---NA---	3.980 down	0.0139
ASS2_C1770	4544	low quality protein: supervillin-like	2.079 down	0.0134
ASS2_C2451	550	---NA---	2.187 down	0.0132
ASS2_C7853	1974	---NA---	2.856 down	0.0124
ASS2_C15020	816	---NA---	2.664 down	0.0119
ASS2_C17614	565	hypothetical protein KGM_17409	4.525 down	0.0117
ASS2_C20696	2360	moderately methionine rich storage protein	168.850 down	0.011
ASS2_C7841	1038	calbindin-32-like isoform x1	2.381 down	0.00964
ASS2_C6231	1071	repeat element protein-	2.352 down	0.00961
ASS2_C21583	270	---NA---	2.141 down	0.00938

ASS2_C16717	924	sodium channel protein type 7 subunit alpha	2.130 down	0.00932
ASS2_C15229	493	---NA---	3.154 down	0.00932
ASS2_C11171	531	hypothetical protein KGM_13152	3.309 down	0.00881
ASS2_C2519	3462	breast carcinoma amplified sequence	2.043 down	0.00741
ASS2_C23934	820	tpa: cuticle protein	37.879 down	0.00722
ASS2_C23995	226	---NA---	71.899 down	0.00651
ASS2_C4190	882	calbindin-32-like isoform x2	2.669 down	0.00627
ASS2_C23972	399	27 kda hemolymph protein	87.795 down	0.00602
ASS2_C19283	556	---NA---	3.319 down	0.00493
ASS2_C18134	2059	gpi-anchor transamidase	4.063 down	0.00485
ASS2_C11852	2748	protein distal antenna	3.435 down	0.00481
ASS2_C3180	2932	beta-glucosidase precursor	2.136 down	0.0035
ASS2_C11784	499	---NA---	3.614 down	0.0027
ASS2_C13030	1786	e3 ubiquitin-protein ligase protein pff1365c-like	5.120 down	0.00261
ASS2_C17564	296	---NA---	3.433 down	0.00208
ASS2_C5191	2220	arylphorin precursor	126.401 down	0.00146
ASS2_C1911	2688	isoform d	3.480 down	0.00142
ASS2_C20725	2325	methionine-rich storage protein	102.093 down	0.000474
ASS2_C23935	1382	arylphorin subunit alpha	231.013 down	0.000264
ASS2_C23991	233	---NA---	112.060 down	3.58E-05
ASS2_C23974	253	---NA---	202.078 down	7.30E-06

Chapter 6

Body odours of parasitized caterpillars give away the presence of parasitoid larvae to their primary hyperparasitoid enemies

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Abstract

Foraging success of parasitoids depends on the utilization of reliable information on the presence of their often, inconspicuous hosts. These parasitic wasps use herbivore-induced plant volatiles (HIPVs) that provide reliable cues on host presence. However, host searching of hyperparasitoids, a group of parasitoids that parasitize the larvae and pupae of other parasitoids, is more constrained. Their hosts do not feed on plants, and often are even concealed inside the body of the herbivore host. Hyperparasitoids recently have been found to use HIPVs of plants damaged by herbivore hosts in which the parasitoid larvae develop. However, hyperparasitoids that search for these parasitoid larvae may be confronted with healthy and parasitized caterpillars on the same plant, further complicating their host location. In this study, we addressed whether the primary hyperparasitoid *Baryscapus galactopus* uses caterpillar body odours to discriminate between unparasitized herbivores and herbivores carrying larvae of parasitoid hosts. We show that the hyperparasitoids made faster first contact and spent a longer mounting time with parasitized caterpillars. Moreover, although the three parasitoid hosts conferred different fitness values for the development of *B. galactopus*, the hyperparasitoids showed similar behavioural responses to caterpillar hosts carrying different primary parasitoid hosts. In addition, a two-chamber olfactometer assay revealed that volatiles emitted by parasitized caterpillars were more attractive to the hyperparasitoids than those emitted by unparasitized caterpillars. Analysis of volatiles revealed that body odours of parasitized caterpillars differ from unparasitized caterpillars, allowing the hyperparasitoids to detect their parasitoid host.

Keywords: *Baryscapus galactopus*, caterpillar body odours, Eulophidae, fourth trophic level, host searching behaviour, Hymenoptera, hyperparasitoid, multi-trophic interactions.

Introduction

Foraging behaviour of herbivores and that of natural enemies underpins much ecological and evolutionary theory, for example evolution of herbivore host-plant range, the preference-performance hypothesis, and predator-prey relationships (Karban & Agrawal 2002; Ode 2006). Foraging is a challenging task for invertebrate carnivores searching for prey that are often inconspicuous. To locate inconspicuous prey, carnivores may use cues that predict prey presence indirectly. For example, parasitoids lay their eggs in or on the bodies of other insects that function as host during the development of the parasitoid larvae (Godfray 1994), and adult female wasps use plant odours emitted in response to feeding damage of their herbivore hosts. These so-called herbivore-induced plant volatiles (HIPVs) differ from volatiles from an undamaged plant and may be specific for the herbivore species feeding on the plant, thereby containing reliable information for parasitoids to locate their host (Vet & Dicke 1992; Dicke 2009; McCormick *et al.* 2012).

Parasitic wasps at the fourth trophic level (hyperparasitoids) that parasitize larvae or pupae of primary parasitoids may even be more constrained than primary parasitoids in locating suitable hosts, since neither the larvae nor the pupae of their primary parasitoid hosts directly feed on the plants (Sullivan 1987). To cope with this problem, hyperparasitoids may rely on HIPVs induced by parasitized caterpillars that differ in composition from HIPVs induced by unparasitized caterpillars (Poelman *et al.* 2012). Moreover, the HIPVs may provide information on the parasitoid species developing inside the herbivore (Poelman *et al.* 2012). This is caused by the abilities of primary parasitoids to manipulate the development, physiology and behaviour of their hosts (Beckage & Gelman 2004; Lemaitre & Hoffmann 2007; Harvey *et al.* 2008; Libersat *et al.* 2009; Thomas *et al.* 2012) and thereby to alter the interaction of their host caterpillar with its food plant (Poelman *et al.* 2011b). Although limited detailed information is available on the physiological changes in caterpillars upon parasitism that result in an altered interaction of their host with the food plant, variation in host manipulation by parasitoid species may result in differentially induced responses in plants by caterpillars in which different species of parasitoids develop. This in turn may result in changes in parasitoid-specific trait-mediated species interactions that are manifested across ecological communities (Poelman *et al.* 2011a). Although HIPVs may provide hyperparasitoids with reliable and detectable cues on host presence, primary hyperparasitoids that parasitize the larvae of parasitoids inside the herbivore hosts may be further constrained when landing on plants infested with parasitized caterpillars. The larval hosts of primary hyperparasitoids are concealed in the bodies of parasitized caterpillars, and these parasitized caterpillars may live and feed on plants adjacent to unparasitized caterpillars (Harvey *et al.* 2012). Besides using HIPVs as

cues for long-range host searching, these hyperparasitoids may have to rely on other cues that come directly from the caterpillars in which their hosts develop after arrival on the herbivore-infested plant. A potential source of information for hyperparasitoids to locate their hosts from a short distance may be herbivore body odours (Weinhold & Baldwin 2011). However, thus far this potential mechanism has not been studied.

Here, we investigated the behaviour of the primary hyperparasitoid wasp *Baryscapus galactopus* Ratzeburg (Hymenoptera: Eulophidae) for its ability to use herbivore-derived odours to locate its host. *Baryscapus galactopus* is an abundant hyperparasitoid in Eurasia and attacks the larval stages of several species of primary parasitioids that develop inside their caterpillar host. As a gregarious koinobiont, *B. galactopus* lays multiple eggs inside a single parasitoid larva, and its larvae develop inside the parasitoid hosts that continue to feed and grow within their own herbivore host (Harvey *et al.* 2013). Eventually, the hyperparasitoids emerge as adult wasps from the pupae of their parasitoid host (Harvey *et al.* 2012). We specifically addressed whether *B. galactopus* discriminates between odours of healthy caterpillars and those that contain its hosts, i.e. parasitized caterpillars, and whether it discriminates between parasitized caterpillars in which different primary parasitoid species develop. We also investigated the fitness correlates of *B. galactopus* when developing in the larvae of three primary parasitoid species: *Cotesia rubecula* Marshall (Hymenoptera: Braconidae), *Hyposoter ebeninus* Gravenhorst (Hymenoptera: Ichneumonidae) and *Cotesia glomerata* L. (Hymenoptera: Braconidae). Finally, we collected volatiles from the headspace of unparasitized and parasitized herbivores to study the differences in body odours.

Materials and methods

Insects

Three parasitoid species that use caterpillars of *Pieris rapae* L. (Lepidoptera: Pieridae) as their host in nature were used in this study. *Cotesia rubecula* and *H. ebeninus* are two solitary endoparasitoids, which lay a single egg per host. Parasitism by *C. rubecula* leads to developmental arrestment of the host in the third or fourth instar of the caterpillar (Harvey *et al.* 1999). Fully developed larvae will emerge and spin a cocoon adjacent to their host. *Hyposoter ebeninus* exhibits a host-regulation pattern similar to *C. rubecula* (Harvey *et al.* 2010). However, unlike *C. rubecula*, *H. ebeninus* larvae consume all host tissues and pupate in the host. In contrast to the two solitary endoparasitoid species, *C. glomerata* is a gregarious endoparasitoid that lays multiple eggs with variable clutch size between 10 to 50 in one host caterpillar. Compared to healthy hosts, caterpillars carrying fully-grown *C. glomerata* larvae are comparable, or even slightly larger in body size, than healthy hosts, although this

depends on the brood size of *C. glomerata* that is developing inside the herbivore (Harvey 2000).

Cultures of the primary parasitoids *C. glomerata, C. rubecula* and their host, the small cabbage white butterfly *P. rapae* were based on insects collected from agricultural fields near Wageningen University, The Netherlands. The culture of *H. ebeninus* was originally collected as cocoons from cabbage fields near the University of Rennes, France (Harvey *et al.* 2010). Hosts and parasitoids were reared on cultivated cabbage plants (*Brassica oleracea* var *gemmifera* cv. Cyrus) in glasshouse compartments (22 ± 1 °C, 50-70% relative humidity, and 16:8 h L:D photoperiod).

The hyperparasitoid *B. galactopus* was originally recovered from *C. glomerata* cocoons collected from experimental fields near Wageningen University, The Netherlands. As endoparasitic koinobiont, the adult female of *B. galactopus* first penetrates the cuticle of a parasitized caterpillar with its ovipositor, and then locates a primary parasitoid larva for oviposition. After hatching of the hyperparasitoid eggs, the larvae feed on haemolymph and fat body of its parasitoid hosts. *Baryscapus galactopus* larvae remain inside the host when host larvae emerge from the caterpillar and pupate. They then kill the host and pupate inside the host cocoon, and several days later adult *B. galactopus* wasps chew holes in and egress from the host cocoons. In order to maintain the culture, we placed two fifth-instar *Pieris. brassicae* caterpillars that had been parasitized by *C. glomerata,* with eight mated female *B. galactopus* wasps in a glass vial for four hours. After hyperparasitism, *P. brassicae* caterpillars were reared on food plants until egression of *C. glomerata* larvae. After egression, the parasitoid larvae immediately spin a cluster of yellowish cocoons adjacent to the caterpillar body. Cocoons of *C. glomerata* were placed in a Petri dish in a climate chamber (22 ± 0.5 °C, 50-70% relative humidity, and 16:8 h L:D photoperiod). Emerged hyperparasitoid wasps were kept in a cage that was away from caterpillars and plants, and were constantly supplied with 10% honey water.

Hyperparasitism by *B. galactopus* was most successful when oviposition happened in the late larval developmental stage of its parasitoid hosts (Harvey *et al.* 2012). Therefore, we selected exclusively for our experiments those parasitized herbivores that were carrying fully-grown primary parasitoid larvae, which means that the caterpillars were in their third instar when parasitized by *C. rubecula* and *H. ebeninus* and in the fifth instar for caterpillars parasitized by *C. glomerata*.

Development of *B. galactopus* in different parasitoid hosts

To study the life history traits of *B. galactopus* developing in three different primary parasitoid hosts, we offered single mated female hyperparasitoid wasps either one fifth instar *P. rapae* caterpillar parasitized by *C. glomerata*, or one late third instar *P. rapae*

caterpillar parasitized by *C. rubecula*, or *H. ebeninus* in a glass vial. Sixty replications were carried out for caterpillars carrying larvae of each primary parasitoid. The female wasp was allowed to oviposit for three hours. Afterwards, parasitized caterpillars were separated from *B. galactopus*, and reared on a food plant until primary parasitoids emerged and pupated. Cocoons of *C. rubecula* and *H. ebeninus* were collected and stored individually in 1 ml Eppendorf tubes that were closed with cotton wool, whereas cocoon clutches of *C. glomerata* were first separated gently with forceps to store each cocoon of a brood in a different tube. The cocoons were placed in a climate cabinet at 22 ± 0.5 °C with 16:8 h L:D photoperiod. We measured fitness-related parameters for *B. galactopus*, including survival rate (percentage of caterpillar hosts that produced hyperparasitoids), egg-to-adult development time, sex ratio, clutch size and fresh weight. Because of the gregarious nature of *C. glomerata*, *B. galactopus* were able to attack multiple parasitoid host larvae within a single caterpillar host. Therefore, we also reported both number of emerging hyperparasitoids per host caterpillar and number of hyperparasitoids per parasitoid host larva. For the solitary parasitoids *C. rubecula* and *H. ebeninus*, the number of emerging hyperparasitoids per host caterpillar was equal to the number of hyperparasitoids per parasitoid host larva. Hyperparasitoid emergence was monitored every four hours to determine the development time from hyperparasitism to adult hyperparasitoid emergence. The cocoons with neither parasitoid nor hyperparasitoid emergence were dissected to determine whether they contained dead parasitoids or hyperparasitoids. Upon eclosion, *B. galactopus* adults were immediately frozen at -20 °C. After this, the wasps were sexed and weighed on a microbalance (accuracy=1 µg; Sartorius AG, Göttingen, Germany).

Behavioural responses of *B. galactopus* to unparasitized caterpillars and caterpillars parasitized by different parasitoids

A no-choice bioassay was carried out to study whether hyperparasitoids respond differently towards healthy caterpillars and caterpillars parasitized by different parasitoid species. One mated naïve female *B. galactopus* and one unparasitized or parasitized caterpillar were placed in a glass Petri dish (9 cm diameter, 1.9 cm height). We provided *B. galactopus* one hour and recorded the time that it spent to make the first contact with the caterpillar. After the first contact, *B. galactopus* started mounting the caterpillar body. In the hour following mounting, we observed the behaviour of *B. galactopus* and recorded the total mounting time. In order to rule out effects of caterpillar body size as a consequence of parasitism by different parasitoid species, we used unparasitized caterpillars from two different stages, i.e. the third larval stage (S-PR or small *P. rapae*) that matched arrestment of caterpillar growth by *C. rubecula* (CR-PR) and *H. ebeninus* (HE-PR), and the fifth larval stage (B-PR or big *P. rapae*) that matched caterpillar size of *C. glomerata*-parasitized *P. rapae* (CG-PR).

Two-chamber olfactometer bioassay for responses of *B. galactopus* to body odours of unparasitized and parasitized caterpillars

We used a newly designed two-chamber olfactometer to further test whether *B. galactopus* can recognize body odours emitted by unparasitized and *C. glomerata*-parasitized caterpillars (Figure 1ab). Each of the two chambers contained either one unparasitized caterpillar (PR) or one parasitized caterpillar (CG-PR), or remained empty (E), testing the preference of hyperparasitoids in the full factorial design of the three treatments. Five mated naïve female *B. galactopus* were released at the centre of olfactometer at the same time. The wasps were allowed one hour to respond to the caterpillar odours and we recorded the number of wasps that had entered each chamber after this time period. In preliminary tests, we observed that *B. galactopus* did not exit once they had entered one of the two chambers.

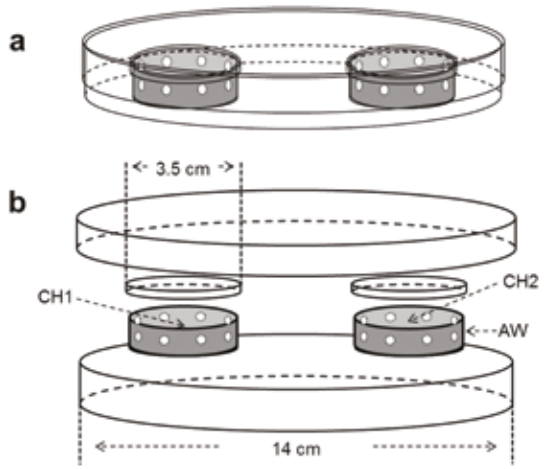

Figure 1. (a) The design of the two-chamber olfactometer. (b) The olfactometer consists of two chambers (CH1 and CH2; 3.5 cm in diameter), with 8 holes (0.3 cm in diameter) in the wall around each chamber, allowing *Baryscapus. galactopus* to enter the chamber. The chamber walls were covered with aluminium foil (AW), so that the hyperparasitoid cannot see the hosts.

Collection of the headspace of the caterpillar's body

To characterize the body odours of healthy and parasitized caterpillars, we collected ten headspace samples from either unparasitized *P. rapae* caterpillars or caterpillars parasitized by *C. glomerata*. All unparasitized and parasitized caterpillars used for headspace collection were in the fifth larval stage. Prior to volatile collection, unparasitized or *C. glomerata*-parasitized caterpillars were transferred from their food plants into a 500 ml glass jar and sealed with a viton-lined glass lid with an inlet and outlet. Each glass jar contained seven *P. rapae* caterpillars from the same treatment. Synthetic air (Linde Gas Benelux B.V., NL) used as a carrier of volatiles was passed through charcoal before flowing into the glass jar containing the caterpillars. Volatiles emitted by the caterpillar were trapped by sucking air out of the glass jar at a rate of 100 ml/min through a stainless steel tube filled with 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) for 2 hours. Immediately after collection, the

Tenax TA cartridges with sample volatiles were dry-purged under a stream of nitrogen (50 ml/min) for 10 min at room temperature (21 ± 2 °C) to remove moisture before storage. The caterpillars used during each sampling were immediately weighed using a microbalance (accuracy = 1 µg; Sartorius AG, Göttingen, Germany). To control for non-caterpillar derived odours, we trapped volatiles from an empty jar and removed compounds found in these samples from further data analysis.

Analysis of volatiles

A combination of Thermo Trace Ultra gas chromatography (GC) and Thermo Trace DSQ quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, USA) was used for the analysis of volatiles associated with the caterpillar body odour. Prior to releasing the volatiles, each sample was dry-purged under a stream of nitrogen (50 ml/min) for 10 min at room temperature (21 ± 2 °C) in order to remove moisture. The volatiles were then thermally released from the Tenax TA (CAMSCO) using an Ultra 50:50 thermal desorption unit (Markes, Llantrisant, UK) at 250 °C for 10 min under a helium flow of 20 ml/min, while re-collecting the volatiles in a cooled solvent trap – Unity (Markes) at 10 °C. Once the desorption process was completed, volatiles were released from the cold trap by fast heating at 40 °C/s to 280 °C, which was then kept for 10 min, while the volatiles were transferred to a ZB-5MSi analytical column [30 m L x 0.25 mm I.D. x 1.00 µm F.T. (Phenomenex, Torrance, CA, USA)], in a splitless mode for further separation. The GC was operated at an initial oven temperature of 40 °C held for 2 min and was then raised at 10 °C/min to a final temperature of 280 °C, where it was held for 4 min under a helium flow of 1 ml/min in a constant flow mode. The DSQ mass spectrometer (MS) was run in a scan mode in a mass range of 35 – 350 amu at 5.38 scans per second and mass spectra were recorded in electron impact ionisation (EI) mode at 70 eV. The temperatures of the MS transfer line and ion source were set to 275 and 250 °C, respectively. Tentative identification of compounds was based on comparison of mass spectra and linear retention indices (LRI) with those in the NIST 2005 and Wageningen Mass Spectral Database of Natural Products mass spectra libraries. We analysed all samples and reference alkanes for the RI in a full scan mode under the same analytical conditions and total ion current (TIC) chromatograms were obtained. A target (single) ion for each compound was selected and used for the measurement of peak area. Volatiles from the synthetic air, empty glass jars, clean Tenax TA adsorbents and the analytical system itself were treated as blank samples and used for corrective measures during analysis.

Statistical analysis

To determine the performance of *B. galactopus* developing in three different parasitoid hosts, the differences in egg-to-adult development time were analysed

with Kaplan-Meier survival test. The differences in sex ratio and number of offspring per host larva were tested with General Linear Model (GLM) one-way ANOVA. We conducted *Pearson's chi-squared test* for percentage host caterpillars that yielded hyperparasitoids. Two-way ANOVA with host parasitoid species and hyperparasitoid sex as main factors were used to statistically analyse adult biomass of *B. galactopus*. Post-hoc multiple comparisons were conducted using Tukey-Kramer tests to reveal differences among means if the models were significant. The differences in first contact time and mounting time of *B. galactopus* with unparasitized caterpillars and caterpillars parasitized by one of three parasitoid species were analysed using one-way ANOVA. *Baryscapus. galactopus* preferences in two-chamber olfactometer bioassays were analysed using *Wilcoxon matched-pair signed-rank test*s.

We used Partial Least Squares Projection to Latent Structures-Discriminant Analysis (PLS-DA) to analyse which of the compounds contributed most to describing the difference in headspace composition between the two caterpillar treatments. The measured peak area for the volatile blends in the different treatments were log-transformed, mean-centred and scaled to unit variance before being analysed using PLS-DA. The results of the analysis are visualized in score plots and loading plots. The score plots reveal the sample structure according to the model components. The loading plots display the contribution of the variables to these components and the relationships among the variables. The program's cross-validation procedure examines the significance of each additional component by comparing the goodness of fit (R^2) and the predictive value (Q^2) of the extended model. Student's T-test analyses were performed on the scores of the first two principle components with two caterpillar treatments. Data on peak area units of the compounds for which the Variable Importance in the Projection (VIP) scores for the PLS-DA were larger than 1, were subjected to Mann–Whitney U tests to examine for significant differences between treatments. All statistical analyses were performed with the statistical software package IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA), except the multivariate data analysis (PLS-DA), which was carried out using the SIMCA P+ version 12.0.1.0 (Umetrics, Umeå, Sweden).

Results

Performance of *B. galactopus* in different parasitoid hosts

To investigate the development of *B. galactopus* in the three different parasitoid hosts, we measured fitness-related traits (Table 1; Figure 2). Forty-six per cent of the hyperparasitized *H. ebeninus* cocoons produced adult *B. galactopus*, whereas 73 and 70 per cent of the *C. glomerata* and *C. rubecula* cocoons, respectively, produced

hyperparasitoids (*Pearson's chi-squared test*, $\chi^2 = 10.033$, df = 2, P = 0.007). Egg-to-adult development time of *B. galactopus* was longer for wasps emerging from *H. ebeninus* than from those emerging from the other two parasitoid species (*Kaplan-Meier test*, $\chi^2 = 15.718$, df = 2, P < 0.001). *Cotesia glomerata* produced *B. galactopus* with highest female:male sex ratio (*GLM*, F = 5.12, P = 0.007). There was no difference in number of *B. galactopus* produced per caterpillar carrying different parasitoid hosts (*GLM*, F = 2.54 P = 0.083). However, there were fewer *B. galactopus* produced in individual cocoons of the gregarious *C. glomerata* than from cocoons of the two solitary parasitoids (*GLM*, F = 83.41, P < 0.001). Female *B. galactopus* wasps had higher fresh weight than males (*GLM*, F = 44.69, P < 0.001) and hyperparasitoids grew larger in *C. glomerata* and *H. ebeninus* than in *C. rubecula* (*GLM*, F = 8.01, P < 0.001; Figure 2).

Table 1. Performance of the hyperparasitoid *Baryscapus. galactopus* in three different primary parasitoid hosts, *Cotesia glomerata*, *Cotesia rubecula* and *Hyposoter ebeninus*. Sixty host caterpillars that parasitized by each parasitoid were tested.

Primary parasitoid hosts	% host caterpillars that yielded hyperparasitoids[x]	Sex ratio (female: male)[y]	Development time (days)[x]		Offspring per host caterpillar[y]		Offspring per host larva[y]	
			Mean	SD	Mean	SD	Mean	SD
Cotesia rubecula	70.0[a]	2.00[a]	19.24[a]	0.17	11.29[a]	5.44	11.29[c]	5.44
Hyposoter ebeninus	46.7[b]	2.44[ab]	20.50[b]	0.28	8.32[a]	6.10	8.32[b]	6.10
Cotesia glomerata	73.3[a]	4.22[b]	19.69[a]	0.14	11.39[a]	6.80	3.53[a]	1.89

[x]: Differences among hosts for fitness-related traits based on pairwise comparisons are indicated with superscript letters.

[y]: Differences among hosts for fitness-related traits based on Tukey-Kramer tests are indicated with superscript letters.

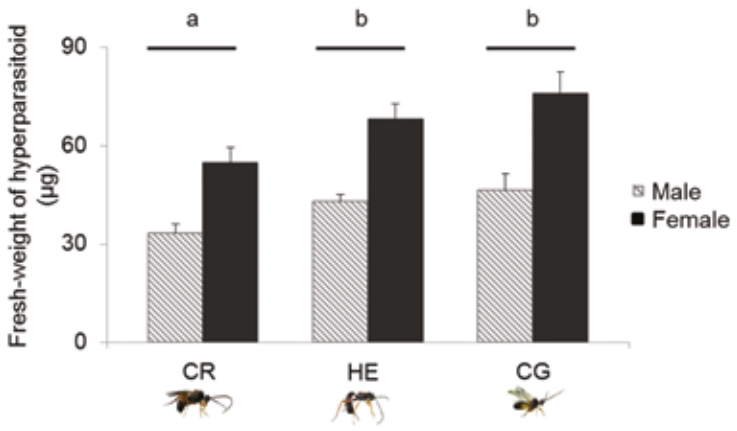

Figure 2. Adult fresh mass of *Baryscapus galactopus* developing in larvae of three parasitoid hosts, *Cotesia rubecula* (CR), *Hyposoter ebeninus* (HE) and *Cotesia glomerata* (CG). Different letters above bars indicate significant differrences (*Tukey-Kramer tests*, P < 0.05).

Table 2. Volatile compounds tentatively identified in the headspace of unparasitized and *Cotesia. glomerata* parasitized *Pieris rapae* caterpillars.Only the compounds that were present in at least 50% of the total samples have been included in analysis. Variable Importance in the Projection (VIP) values for the PLS-DA are given. Bold face type scores are higher than 1 and are most influential for separation of the treatments. P-values indicate the differences between treatments for each compound base on Mann–Whitney U pairwise comparisons.

No.	Compound	Class	$LRI_{Exp.}$ [a]	$LRI_{Lit.}$ [b]	Unparasitized *P. rapae*[d]	Parasitized *P. rapae*[d]	P-value	VIP Score	Previous records[e]
1	3-Pentanol	Alcohol	693	710	18.33 (2.35)	8.64 (2.11)	0.005	**1.22**	Plant & frass
2	(Z)-3-Hexen-1-ol	Alcohol	857	857	3.19 (0.49)	2.97 (0.69)	0.496	0.95	Plant & frass
3	Cyclohexanol	Alcohol	889	881	5.86 (0.54)	6.55 (1.31)	0.762	0.99	–
4	2-Butoxyethanol	Alcohol	907	909	4.40 (0.59)	2.23 (0.38)	0.013	**1.13**	–
5	Benzyl alcohol	Alcohol	1043	1042	14.09 (5.70)	4.16 (0.71)	0.049	0.96	–
6	Methylthioacetaldehyde	Aldehyde	760	755[c]	7.47 (2.46)	4.04 (1.12)	0.406	0.91	Frass
7	(E)-2-Hexenal	Aldehyde	855	855	1.27 (0.16)	1.18 (0.25)	0.364	0.92	Plant & frass
8	Methyl salicylate	Ester	1215	1208	3.04 (2.38)	0.32 (0.23)	0.031	0.76	Plant
9	2,3-Butanedione	Ketone	588	586	53.35 (5.69)	160.46 (35.97)	0.016	**1.42**	Frass
10	3-Butenenitrile	Nitrile	652	658	3.05 (1.15)	0.22 (0.12)	0.007	**1.14**	Plant[f]
11	4-Methylthiobutyronitrile	Nitrile	1092	1061[c]	7.88 (1.07)	3.85 (0.90)	0.013	**1.17**	Frass
12	Dimethyl disulfide	Sulfide	747	746	294.09 (69.82)	83.72 (14.86)	0.008	**1.29**	Plant & frass
13	Dimethyl trisulfide	Sulfide	988	989	26.67 (12.10)	12.36 (3.42)	0.496	0.53	Plant & frass
14	Dimethyl sulfone	sulfone	916	915	10.91 (1.36)	6.48 (1.57)	0.016	**1.07**	Plant
15	Unknown	NA	NA	NA	4.58 (1.80)	4.02 (2.02)	0.29	0.38	–
16	Unknown	NA	NA	NA	2.24 (0.63)	1.97 (0.66)	0.597	0.54	–

[a]: $LRI_{Exp.}$: linear retention indices experimentally obtained on a ZB-5MSi analytical column.

[b]: $LRI_{Lit.}$: linear retention indices obtained from literature [NIST 2005, Wageningen University Mass Spectral library, and The Pherobase (http://www.pherobase.com/database/kovats/kovats-index.php) on a column with (5%-Phenyl)-methylpolysiloxane stationary phase or equivalent.

[c]: $LRI_{Lit.}$: LRI on a 100% polydimethylsiloxane (PDMS) or equivalent stationary phase (Rochat *et al.* 2007; Iranshahi 2012).

[d]: Volatile emissions are given as mean peak area (SE) per g fresh weight of caterpillar divided by 10^3.

[e]: We compared the volatile compounds identified in the headspace of caterpillars with compounds previously reported in volatile profiles of *Brassica oleracea* var *gemmifera* cv. Cyrus plants (Gols *et al.* 2011; Poelman *et al.* 2012; Soler *et al.* 2012) and *Pieris rapae* caterpillar frass (Agelopoulos *et al.* 1995), for caterpillar body derived compounds.

[f]: Our unpublished data of volatile profiles of *Brassica oleracea* var *gemmifera* cv. Cyrus plants.

NA: Not Available.

Hyperparasitoid behaviour in response to parasitized and unparasitized caterpillars

Within one hour of release, *B. galactopus* responded in 99% of the cases to the healthy and parasitized caterpillars offered in a no-choice assay in a Petri dish. The hyperparasitoids were faster in making first contact with parasitized caterpillars compared to unparasitized caterpillars (*GLM*, $F = 7.97$, $P < 0.001$) and did not respond differently to different instars of caterpillars or species of parasitoid developing inside the caterpillar (Figure 3a). *Baryscapus galactopus* spent longer time mounting on parasitized than on healthy caterpillars regardless of the instar of the caterpillar (*GLM*, $F = 10.07$, $P < 0.001$). On all three types of parasitized caterpillars, most *B. galactopus* were still mounting after one hour when the experiment was stopped (Figure 3b).

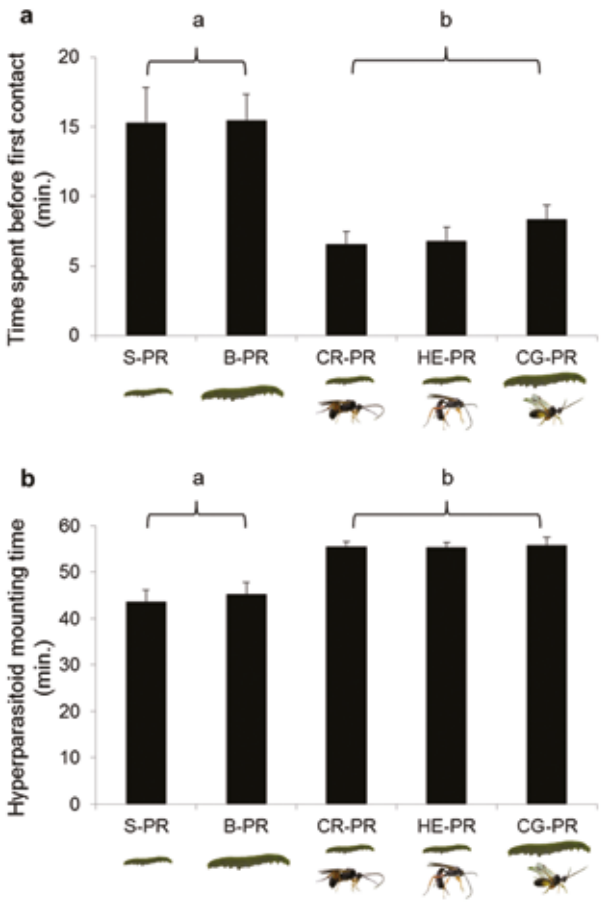

Figure 3. Mean (a) time until first contact with the herbivore host *Pieris rapae*, (b) mounting time of *B. galactopus* on caterpillar within one hour after first contact was made for small (S-PR) and large (B-PR) healthy caterpillars, and caterpillars parasitized by *Cotesia rubecula* (CR-PR), *Hyposoter ebeninus* (HE-PR) and *Cotesia glomerata* (CG-PR). Different letters above bars indicate significant differences (*Tukey-Kramer tests*, $P < 0.05$). Sample size: n = 40, for first contact; n=30, for mounting time.

Two-chamber olfactometer bioassay

In total 520 *B. galactopus* females were tested in two-choice assays; 40% of the tested wasps made choices within one hour. The wasps preferred odours released from chambers that contained a healthy or parasitized herbivore over odours from empty chambers (*Wilcoxon matched-pair signed-rank test*, unparasitized *P. rapae*: Z = -4.118, P < 0.001; *C. glomerata* parasitized *P. rapae*: Z = -2.743, P = 0.006). When the body odours of both unparasitized and parasitized caterpillars were offered, the wasps were more attracted by body odours of parasitized than unparasitized caterpillars (*Wilcoxon matched-pair signed-rank test,* Z = -2.905, P = 0.004; Figure 4).

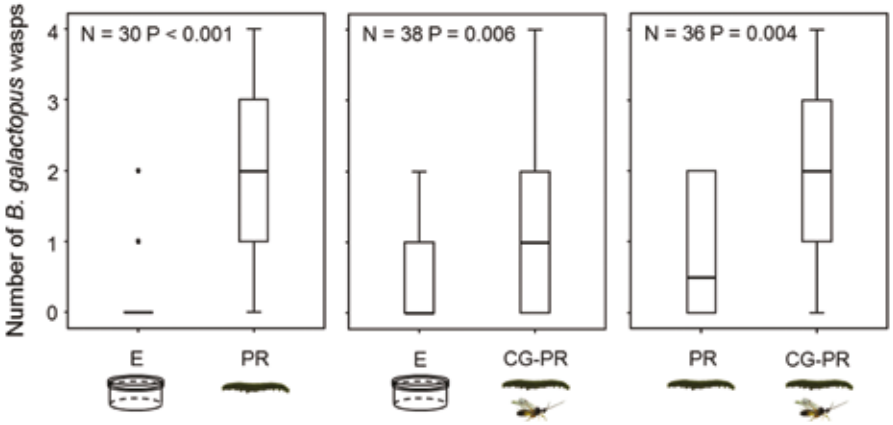

Figure 4. Number of *B. galactopus* out of 5 wasps that entered one of the two chambers in olfactometer. Empty chamber (E), unparasitized *Pieris. rapae* (PR), *Cotesia. glomerata* parastized *P. rapae* (CG-PR). N = the number of replicates that each consist of releasing 5 *B. galactopus* into the olfactometer.

Caterpillar body odour

Since *B. galactopus* showed similar behavioural responses to the three types of parasitized caterpillars, we chose *C. glomerata*-parasitized *P. rapae* caterpillars for further body odour analysis. Analysis of the volatile blends of the caterpillar body showed that body odour of parasitized caterpillars differs from that of unparasitized caterpillars. In the PLS-DA score plot, the samples of unparasitized caterpillars and caterpillars parasitized by *C. glomerata* were clearly separated from each other based on the volatiles emitted (PCs; model statistics: $R^2X = 0.495$, $R^2Y = 0.85$ and $Q^2 = 0.62$; *Student's t-test* on scores of first PC: t = 5.80, df = 18, P < 0.001, and second PC: t = 2.28, df = 18, P = 0.035; Figure 5a). Sixteen different compounds were tentatively identified in unparasitized or *C. glomerata*-parasitized *P. rapae* caterpillars (Table 2). Seven compounds strongly contributed to the differences among treatments, as indicated by VIP values larger than 1 (Table 2). Six of those compounds were emitted in lower amounts from *C. glomerata*-parasitized caterpillars (Table 2, Figure 5b). Interestingly, 2,3-butanedione was the only compound that had a significantly

higher concentration in volatile blends of *C. glomerata*-parasitized caterpillars than unparasitized caterpillars (Table 2, Figure 5b).

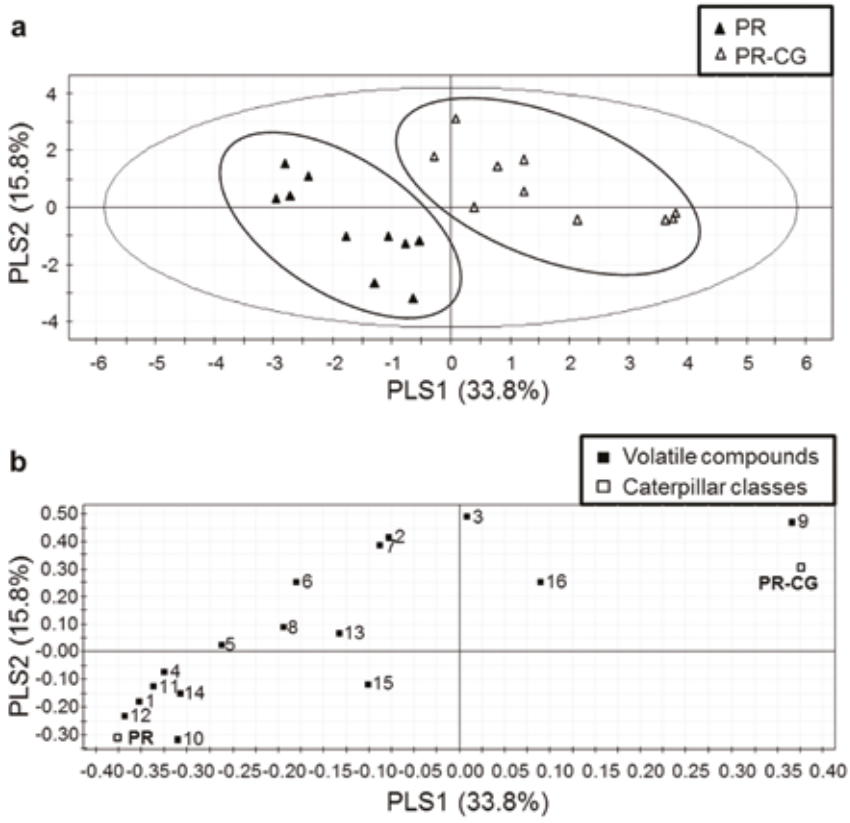

Figure 5. PLS-DA (Projection to Latent Structures-Discriminant Analysis) of quantities of volatile compounds produced by unparasitized *Pieris. rapae* caterpillars (PR) or caterpillars parasitized by *Cotesia. glomerata* (PR-CG). The score plot (a) visualizes the structure of the samples according to the first two PLS components with the explained variance in brackets. The Hotelling's T^2 ellipse confines the confidence region (95%) of the score plot. The loading plot (b) defines the contribution of each of the volatile compounds to the first two principal components. For compound identity see Table 2.

Discussion

Although hyperparasitoids may exert "top-down" control of terrestrial herbivorous arthropod populations by parasitoids, as well as on the structure of the arthropod community (Rosenheim 1998), little is known about foraging behaviour of these insects, nor the cues used during host searching (but see Sullivan & Volkl 1999; Volkl & Sullivan 2000; Poelman *et al.* 2012; Whiteman 2012). *Baryscapus galactopus* is the dominant primary hyperparasitoid species in *Brassica*-associated insect communities

(Tanaka *et al.* 2007; Poelman *et al.* 2012), and is the enemy of several species of primary parasitoids. Here, we show that three of its parasitoid hosts (*C. rubecula*, *H. ebeninus* and *C. glomerata*) largely differ in their fitness value for *B. galactopus*. When *B. galactopus* developed in *C. glomerata,* they achieved a higher survival rate, female:male sex ratio and adult fresh-mass, and a shorter egg-to-adult development time (Table 1; Figure 2). The hyperparasitoids performed most poorly on *H. ebeninus*, where successful hyperparasitism was lowest, indicating that it is a less suitable host for *B. galactopus* than *C. glomerata* and *C. rubecula*. The hyperparasitoids that we used in the current study were reared on *C. glomerata*, which may potentially influence the performance of the hyperparasitoids on different parasitoid hosts. Future studies may consider investigating the effects of hyperparasitoid rearing history on their performance on different parasitoid hosts.

The results of the no-choice bioassays indicate that *B. galactopus* differs in behaviour when encountering *P. rapae* caterpillars which are either healthy or parasitized by different species of primary parasitoids. Insects use various types of information for locating and accepting a host, such as visual, olfactory, gustatory and mechano-sensory cues (Schoonhoven *et al.* 2005). It has been shown that hyperparasitic wasps are attracted by herbivore-induced plant volatiles (Dicke 2009). In addition, host suitability may also affect the foraging behaviour and host preference of hyperparasitoids (Buitenhuis *et al.* 2004; Buitenhuis *et al.* 2005). Our results with *B. galactopus*, which made faster first contact with parasitized herbivores, suggests that hyperparasitoids also sense the changes in body odours of caterpillars in which parasitoid larvae are present (Figure 3a). This was also demonstrated with further two-chamber olfactometer bioassays in which *B. galactopus* showed higher preferences for body odours emitted by parasitized herbivores than body odours of unparasitized herbivores (Figure 4). Moreover, hyperparasitoids also spent longer time mounting on parasitized caterpillars, indicating that mechano-sensory cues of caterpillars may change due to parasitism as well. These mechano-sensory cues are probably used by hyperparasitoids during mounting of the caterpillar body to precisely locate their host larvae developing inside caterpillars. The longer mounting time of hyperparasitoids may also result from chemicals in the parasitized caterpillars that arrest the hyperparasitoids. Interestingly, although the primary parasitoid hosts used in this study vary in certain aspects of their life histories (Harvey *et al.* 1999; Harvey 2000; Harvey *et al.* 2010), such as in host manipulation (Poelman *et al.* 2011b), and quality (Poelman *et al.* 2012) (Table 1), *B. galactopus* responded similarly in making first contact and mounting on *P. rapae* caterpillars parasitized by the different parasitoids. On the one hand, this suggests either that the hyperparasitoids do not sense differences in body odours of caterpillars containing different species of parasitoid larvae, or else they treat those possible hosts in the same way to maximize

opportunities for oviposition. On the other hand, we might be able to observe differences in mounting time of *B. galactopus* in response to different parasitoid hosts if we had offered a longer mounting period to the hyperparasitoids. This is because most of the hyperparasitoids were still in the process of mounting the caterpillars carrying the different parasitoids at the end of one hour of observation. In order to reveal host preference by hyperparasitoids, additional two-choice assays with each parasitoid treatment are required in future studies. Nevertheless, our data show that primary hyperparasitoids have evolved to respond to herbivore body odours as detectable and reliable cues that indicate the presence of primary parasitoid hosts developing inside the herbivore. This is likely to have evolved as an adaptive mechanism that enables an adult female primary hyperparasitoid to distinguish between healthy and parasitized caterpillars that share the same individual food plant. Females that are able to immediately distinguish between these two host 'types' will waste less time mounting and probing unsuitable (= unparasitized) caterpillars.

To further support the role of caterpillar odours, we have analysed the volatile headspace of caterpillar bodies to characterize the differences in body odour. Our PLS-DA plot shows that volatile profiles of unparasitized and parasitized *P. rapae* caterpillars are clearly different. We compared the 16 compounds tentatively identified from caterpillar bodies to previous studies on volatile analysis of *P. rapae* caterpillar frass (Agelopoulos *et al.* 1995) and *B. oleracea* var *gemmifera* cv. Cyrus plants (Gols *et al.* 2011), in order to identify the potential sources of these compounds. Among the 16 compounds, nine have previously been identified in volatile blends derived from plants or caterpillar frass, or from both sources (Table 2). The caterpillars that were used for body odour collection were removed from host plants just before the volatile collection. Moreover, while collecting caterpillar body odours, caterpillars also produced frass. Therefore, the volatile blends that we collected might contain both plant- and caterpillar frass-derived compounds. *Baryscapus galactopus* probably uses plant-derived volatiles as detectable cues for host searching in complex habitats (Poelman *et al.* 2012; Poelman *et al.* 2013). Yet, we cannot rule out that the hyperparasitoids use volatiles emitted by caterpillar frass only as proximate cues. In total, eight caterpillar-associated volatile compounds were emitted in lower amounts from *C. glomerata*-parasitized *P. rapae*. Interestingly, 2,3-butanedione was the only compound which was found in *C. glomerata*-parasitized *P. rapae* at higher levels, and contributed most to the difference between healthy and parasitized herbivores as indicated by having the highest VIP value in the PLS-DA (Table 2). However, the potential role of this compound in *B. galactopus* host searching behaviour still remains to be elucidated in future studies. Some compounds, such as 2-butoxyethanol, were measured in higher amounts in unparasitized *P. rapae* than in parasitized caterpillars and may further allow hyperparasitoids to discriminate between parasitized and unparasitized

caterpillars. The origin of 2-butoxyethanol may be the herbivore itself since it has not been reported in volatiles of *Brassica* plants or caterpillar frass (Agelopoulos *et al.* 1995; Gols *et al.* 2011; Poelman *et al.* 2012; Soler *et al.* 2012), but we cannot exclude the possibility that caterpillars acquired the compound from materials used for insect or plant rearing. So far, we have analysed headspace volatiles of unparasitized and *C. glomerata*-parasitized caterpillars. Including headspace analysis of the other two parasitoid treatments (*C. rubecula* and *H. ebeninus*) in future studies may provide a better understanding of the foraging cues used by hyperparasitoids.

Although primary parasitoid larvae are concealed within their herbivore host and thereby may seem inconspicuous to their enemies, their feeding inside the caterpillar causes variation in HIPVs that may reliably give away its presence to hyperparasitoid enemies (Dicke 2009). Our current study shows that parasitoid larvae give away their presence even further through changes in body odours of caterpillars in which they develop, allowing for the evolution of finely-tuned foraging behaviours of their enemies in the fourth trophic level. In addition to a suite of behavioural changes that parasitoids induce in their herbivorous hosts (Libersat *et al.* 2009), further studies are needed to investigate the physiological changes in herbivores due to parasitism, in order to better understand the community-wide implications for multitrophic interactions. Beyond the multitrophic interactions mediated by herbivore body odours of the current study, the extended phenotype of parasitoids that influences herbivores directly or plants indirectly may profoundly impact ecological processes (Utsumi 2011; Kaplan 2012).

Acknowledgements

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Chapter 7

Intrinsic competition between primary hyperparasitoids of the solitary endoparasitoid *Cotesia rubecula*

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Abstract

In nature, competitive interactions occur when different species exploit similar niches. Parasitic wasps (parasitoids) often have narrow host ranges and need to cope with competitors that use the same host species for development of their offspring. When larvae of different parasitoid species develop in the same host, this leads to intrinsic and often contest competition. Thus far most studies on intrinsic competition have focused on primary parasitoids. However, competition among hyperparasitoids, parasitic wasps that use primary parasitoids as a host, has been little studied. Here, we investigated intrinsic competition between two primary koinobiont hyperparasitoids, the gregarious *Baryscapus galactopus* and the solitary *Mesochorus gemellus* that lay their eggs in primary parasitoid larvae of *Cotesia rubecula* while those in turn are developing inside the body of their herbivore host, *Pieris rapae*. Our aims were to identify: 1) which hyperparasitoid is the superior competitor, and 2) whether oviposition sequence affects the outcome of intrinsic competition. Our results show that *B. galactopus* won 70 % of contests when the two hyperparasitoids parasitized the host at the same time and 90% when *B. galactopus* oviposited first. When *M. gemellus* had a 48h head start, the two hyperparasitoids had an equal chance to win the competition. This suggests that *B. galactopus* is an intrinsically superior competitor to *M. gemellus*. In addition, the outcome of competition is affected by time lags in oviposition events. In contrast to what has been reported in the literature for primary parasitoids, we found that a gregarious hyperparasitoid species had a competitive advantage over a solitary species.

Keywords: intrinsic competition, insect parasitoid, primary hyperparasitoid, contest competetion, *Baryscapus galactopus*, *Mesochorus gemellus*.

Introduction

It has long been recognised that individual fitness is optimized through the production of large numbers of progeny that in turn also produce many offspring (Stearns 1992). Given that food resources for many consumers are patchily distributed or ephemeral, constraints on diet can also have a major effect on fitness. Many organisms abandon their progeny immediately after laying eggs. For these species, the developmental success of their offspring not only depends on the quantity and quality of their diet, but may also be affected by other organisms that exploit the same resource (Hairston *et al.* 1960; Polis & Holt 1992; Mayhew 1997; Poelman & Dicke 2007). If resources are limiting, this can lead to competitive interactions among individuals, and can generate two main outcomes. Competing organisms can engage in scramble competition (through resource partitioning) when all competitors equally ration the finite resources, resulting in decreased fitness for all competitors (Royle *et al.* 2002; Harvey *et al.* 2013). Alternatively, in contest competition a successful competitor monopolizes all of the resources it requires for survival and reproduction and there is no room for resource sharing (Sterck *et al.* 1997; Harvey *et al.* 2013).

Parasitoid wasps lay their eggs in or on the bodies of other insects and their larvae complete their development by exclusively feeding on the host tissues whereas the adults are free-living (Godfray 1994). Immature parasitoid development is dependent on the resources contained within an individual host, and as a result they are intense selection to optimize the exploitation and allocation of these resources to different, and often competing fitness functions (Sequeira & Mackauer 1993; Harvey 2005). Many parasitoids, in particular those developing inside hosts that are challenged by the host's immunological defences (Strand & Pech 1995), have narrow host ranges and some species of parasitoids may even attack only a single species of host in nature (Godfray 1994; Hawkins 1994). Since host resources are limited, there is little capacity for resource sharing among the progeny of different parasitoid species. Therefore, to maximize reproductive success, parasitoids need not only to overcome a suite of environmental constrains to locate hosts, they may also need to be effective competitors in inter or intra-specific competition (Hochberg 1991; Iwao & Ohsaki 1996; Tian *et al.* 2008; Mohamad *et al.* 2015). Adult parasitoids compete extrinsically when searching for and exploiting hosts, whereas their larvae compete intrinsically when multiple individuals develop in the same host (Force 1974; De Moraes *et al.* 1999; Cusumano *et al.* 2012; Harvey *et al.* 2013). Solitary parasitoids that lay a single egg per host often have larvae that kill or suppress competitors and are thus involved in contest competition in which only a single competitor eventually survives (Fisher 1961, 1963). For gregarious parasitoids that lay multiple eggs per host, scramble competition is the norm in which even larvae of two species may successfully develop inside the same host (Dorn & Beckage 2007; Magdaraog *et al.* 2012).

Several hypotheses have indicated that the outcome of interspecific competition between parasitoids can be affected by various factors. For extrinsic competition, the outcome can be affected by species differences in host-searching efficiency, reproductive capacity (i.e. egg number), as well as phenological synchronization among different parasitoid species with the host (Tumlinson *et al.* 1993; Lei & Hanski 1998; Cronin 2007; Cusumano *et al.* 2012; Magdaraog *et al.* 2013). By contrast, the outcome of intrinsic competition is often influenced by parasitoid growth rate, solitary or gregarious life history, developmental stage of the host, the order of oviposition events and host quality (Tillman & Powell 1992; van Nouhuys & Punju 2010; Harvey *et al.* 2013; Poelman *et al.* 2014).

Thus far, intrinsic competition has been mostly studied among primary parasitoids (Harvey *et al.* 2013), largely ignoring the fact that food chains involving plants, herbivores and parasitoids go to the fourth trophic levels and even higher (Harvey *et al.* 2009b). For example, many primary parasitoids are themselves attacked by hyperparasitoids (Sullivan 1987). The hyperparasitic strategy probably evolved from primary parasitism and has been very successful, with some primary parasitoids harbouring a large number of hyperparasitoids (Sullivan & Volkl 1999; Poelman *et al.* 2013). According to the host developmental stages that hyperparasitoids are attacking, two groups of hyperparasitoids have been described. Secondary hyperparasitoids (ectophagous) attack pupae of their hosts, whereas primary hyperparasitoids (endophagous) oviposit in the larvae of their hosts (van Nouhuys & Punju 2010; Harvey *et al.* 2012; Magdaraog *et al.* 2012; Poelman *et al.* 2012; Zhu *et al.* 2014b). Secondary hyperparasitoids are primarily idiobionts that attack non-growing host stages such as eggs or pupae or else hosts that are paralyzed preceding oviposition, whereas primary hyperparasitoid are usually koinobionts that allow the host to continue feeding and growing during parasitism (Askew & Shaw 1986). Primary hyperparasitism is a complex process whereby females of the primary hyperparasitoid must first penetrate the cuticle of a parasitized caterpillar with their ovipositor, and then locate a primary parasitoid larva in the caterpillar for oviposition. After hatching of the hyperparasitoid eggs, the larvae feed on haemolymph and fat body of its parasitoid host. Larvae of primary hyperparasitoids remain inside the host when the parasitoid host larvae emerge from the caterpillar to pupate. They then kill their parasitoid host and pupate inside the host cocoon, and several days later adult hyperparasitoids chew holes in the host cocoons and egress from them.

Primary hyperparasitoids are often constrained in locating their inconspicuous hosts that are developing inside the herbivore host (Zhu *et al.* 2014b). Moreover, they may frequently encounter competitors as it is not uncommon for two or more hyperparasitoid species (both primary and secondary) to emerge from a single clutch of cocoons of a gregarious parasitoid (Poelman *et al.* 2012; Poelman *et al.* 2013). This suggests that hyperparasitoids may be frequently involved in intrinsic competition. Therefore, when

intrinsic levels of competition are high, selection may favour the evolution of traits in hyperparasitoids that enable them to kill rivals for host resources. Several studies have examined competition between hyperparasitoids, but these were based exclusively on secondary hyperparasitoids (Harvey *et al.* 2009c; Harvey *et al.* 2011b). To the best of our knowledge, competition between primary hyperparasitoids has not been investigated.

In this study, we investigated intrinsic competition between two primary koinobiont hyperparasitoids, *Baryscapus galactopus* Ratzeburg (Hymenoptera: Eulophidae) and *Mesochorus gemellus* Holmgren (Hymenoptera: Ichneumonidae). Both species are important primary hyperparasitoids in the food webs involving *brassica*ceous plants and their associated consumers. In the field, *Brassica* plants are often attacked by caterpillars of a specialist herbivore, the small cabbage white butterfly, *Pieris rapae* L. (Lepidoptera: Pieridae) (Harvey *et al.* 1999; Harvey 2000; Poelman *et al.* 2008a). The primary parasitoids *Cotesia rubecula* Marshall (Hymenoptera: Braconidae) and *C. glomerata* L. (Hymenoptera: Braconidae) are natural enemies of *Pieris* caterpillars, which are in their turn used as common hosts by both *B. galactopus* and *M. gemellus* (Poelman *et al.* 2012; Poelman *et al.* 2013). *B. galactopus* is a gregarious hyperparasitoid that lays up to 30 eggs in individual host larvae (Harvey *et al.* 2012; Zhu *et al.* 2014b), whereas *M. gemellus* is solitary. The main questions that we addressed here were: 1) which primary hyperparasitoid species is superior in intrinsic competition, the gregarious *B. galactopus* or the solitary *M. gemellus*, and 2), does the sequence of hyperparasitism affect the outcome of intrinsic competition among hyperparasitoids.

Materials and methods

Insects

The two primary hyperparasitoid species used in this study, *B. galactopus* and *M. gemellus*, were originally recovered from *C. glomerata* cocoons collected from experimental fields near Wageningen University, The Netherlands (Poelman *et al.* 2012). *B. galactopus* is a gregarious hyperparasitoid that lays up to 30 eggs per host and was reared exclusively in *C. glomerata* for less than ten generations, following the protocol described in Harvey *et al.* (2012) and Zhu *et al* (2014). *Mesochorus. gemellus* is a solitary hyperparasitoid that lays a single egg per oviposition event. We were not able to establish a stable culture of *M. gemellus* in our laboratory. Therefore, the *M. gemellus* hyperparasitoids used in this study were newly emerged wasps from field-collected *C. glomerata* cocoons. Both hyperparasitoid species were kept in cages that were stored in a climate cabinet (22 ± 0.5 °C, 50-70% relative humidity, and 16:8 h L:D photoperiod) away from caterpillars and plants, and were *ad libitum* supplied with 10% honey water.

To study intrinsic competition between the hyperparasitoids, we used *C. rubecula* that is a common host of both hyperparasitoid species. *C. rubecula* is a solitary koinobiont endoparasitoid, which lays a single egg per caterpillar host. To prepare *C. rubecula*-parasitized caterpillars, we offered late first-instar *P. rapae* larvae individually to mated female wasps. Parasitized caterpillars were reared on cultivated cabbage plants (*Brassica oleracea* var *gemmifera* cv. Cyrus) in a glasshouse compartment (22 ± 1 °C, 50-70% relative humidity, and 16:8 h L:D photoperiod) until hyperparasitism. Under these conditions, parasitoid larvae required approximately eight days to complete their larval stages and to emerge from their host body to spin a cocoon. It has been shown that hyperparasitism by *B. galactopus* is most successful when oviposition occurs in the late larval developmental stages of its primary parasitoid host (Harvey *et al.* 2012). Therefore, we selected 24 hours before larval egression (= emergence) of *C. rubecula* larvae (7-day old parastized caterpillar carrying a fully-grown parasitoid larva) as a normal hyperparasitism time point and included an early hyperparasitsm treatment by offering hyperparsitoid-parasitized caterpillars of 5 days old that were 72 hours before emergence of *C. rubecula* larvae.

Experimental design

In order to test the outcome of interspecific intrinsic competition as well as a potential competitive advantage by a head start, we included three multi-hyperparasitism treatments: 1) hyperparasitism by the two hyperparasitoids simultaneously (less than 10 min. difference) at 24 h before larval egression of *C. rubecula* from the host caterpillar (24MG-24BG; N = 56); 2) hyperparasitism by *M. gemellus* at 72 hours and *B. galactopus* at 24 h before larval egression of *C. rubecula* (72MG-24BG; N = 45); 3) hyperparasitism by *B. galactopus* 72 hours and by *M. gemellus* 24 h before larval egression of *C. rubecula* (72BG-24MG; N = 45). In addition, we also used four control treatments, including hyperparasitism by *B. galactopus* at 72 (72BG; N = 45) hours or 24 h (24BG; N = 57), or by *M. gemellus* at 72 h (72MG; N = 45) or 24 h (24MG; N = 57), before larval egression of *C. rubecula*.

Experimental procedure

Individual hyperparasitism by *B. galactopus* or *M. gemellus* was performed in a glass vial, by offering single *C. rubecula*-parasitized *P. rapae* caterpillars to one mated female hyperparasitoid. For *M. gemellus*, hyperparasitism was considered successful when a clear penetration of the ovipositor into the caterpillar body was observed. Oviposition by *B. galactopus* requires more time than for *M. gemellus*, which includes making first contact with the herbivore host, mounting and actual egg deposition (Zhu *et al*., 2014). Therefore, we exposed larvae of *P. rapae* parasitized by *C. rubecula* for one hour to female *B. galactopus* for oviposition. After hyperparasitism, herbivore hosts from

different treatments were returned to separate cages per treatment and were allowed to continue feeding on food plants until *C. rubecula* larvae egressed from the host caterpillars. Individual *C. rubecula* cocoons were collected from the rearing cages, stored in 1.5 ml Eppendorf tubes covered with cotton wool, labelled for the respective hyperparasitism treatment and kept at room temperature (20 ± 1 ºC, 50-70% relative humidity, and 16:8 h L:D photoperiod). Upon eclosion of the wasps, we recorded the species identity (the 'winner' of the competition) and anesthetized the hyperparasitoids with CO_2 to measure their fresh weight on a microbalance (accuracy=1 µg; Sartorius AG, Göttingen, Germany). We also recorded other fitness-related traits, including egg-to-adult development time, sex ratio and clutch size. Hyperparasitoid emergence was monitored every four hours to determine the development time from hyperparasitism to adult hyperparasitoid emergence. Cocoons where neither a parasitoid nor a hyperparasitoid emerged were dissected to determine whether they contained dead parasitoids or hyperparasitoids.

Statistical analysis

The effects of hyperparasitism treatments on host parasitoid mortality and intrinsic competition between hyperparasitoids were analysed using Pearson's chi-squared tests. Pair-wise comparisons using Pearson's chi-squared tests were conducted to reveal the differences among treatments. Two-tailed binomial tests were used to analyse the differences in winning the intrinsic competitions between the two hyperparasitoid species within each multi-parasitism scheme. The differences in egg-to-adult development time between both hyperparasitoid species were analysed with a Kaplan-Meier survival test. Within a hyperparasitoid species, the differences in sex ratio, clutch size and fresh weight of hyperparasitoids were analysed with one-way ANOVAs. Post-hoc multiple comparisons were conducted using Tukey-Kramer tests to reveal differences among means if the models were significant. All statistical analyses were performed with the statistical software package IBM SPSS Statistics 19 (SPSS Inc., Chicago, IL, USA).

Results

Mortality of the parasitized caterpillar did not differ significantly across the seven hyperparasitism treatments (*Pearson's chi-squared test*, $\chi^2 = 6.174$, df = 6, P = 0.40; Figure 1A). However in comparison to mortality in response to hyperparasitism by a single hyperparasitoid species, multi-hyperparasitism, and in particular in the 72BG-24MG combination, resulted in higher host pupal mortality in which neither the primary parasitoid nor hyperparasitoid adults emerged from the cocoons (*Pearson's chi-squared test*, $\chi^2 = 15.487$, df = 6, P = 0.017; Figure 1B).

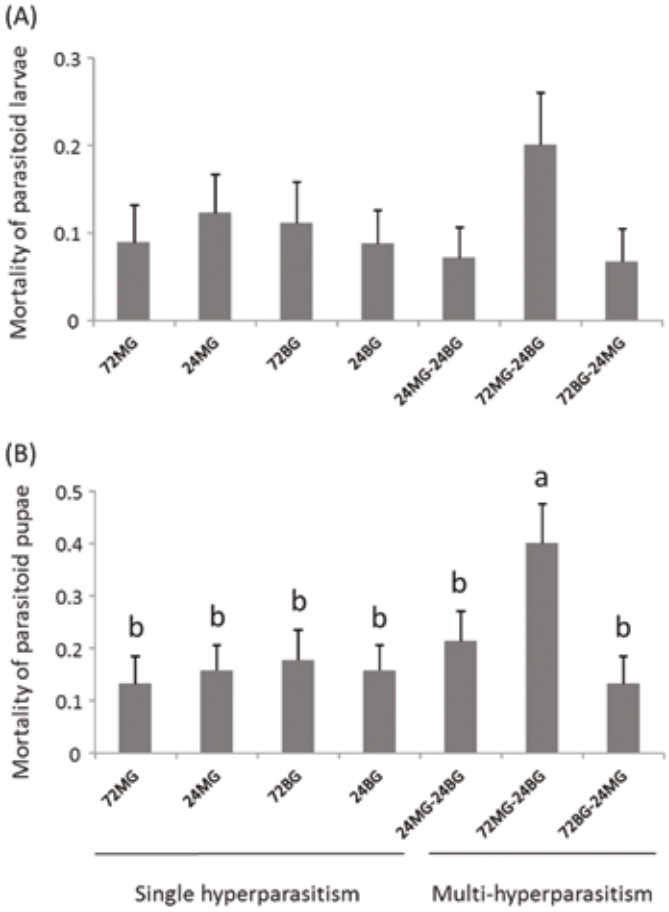

Figure 1. Effects of hyperparasitism treatments on host mortality, (A) mortality of larvae of parasitoid host, (B) mortality of pupae of parasitoid host. 72MG & 24 MG: hyperparasitsm by *Mesochorus gemellus* at 72 h or 24 h before emergence of *C. rubecula*, respectively; 72BG & 24BG: hyperparasitism by *Baryscapus galactopus* at 72 h or 24 h before emergence of *C. rubecula*, respectively; 24MG-24BG: hyperparasitism by *M. gemellus* and *B. galactopus* simultaneously at 24 h before emergence of *C. rubecula*; 72MG-24BG: hyperparasitism by *M. gemellus* at 72 hours and *B. galactopus* at 24 hours before emergence of *C. rubecula*; 72BG-24MG: hyperparasitism by *B. galactopus* at 72 hours and by *M. gemellus* at 24 hours before emergence of *C. rubecula*. Different letters above bars indicate differences (*Pearson's chi-squared tests*, $P < 0.05$).

The three multi-hyperparasitism treatments used here significantly affected the outcome of intrinsic competition between *B. galactopus* and *M. gemellus* (*Pearson's chi-squared test*, $\chi^2 = 8.012$, df = 2, P = 0.018; Figure 2). *Baryscapus galactopus* was more competitive, winning about 70% of the competitions when both hyperparasitoids oviposited simultaneously (binomial test, P = 0.024). Moreover, the time points of oviposition by both hyperparasitoids also influenced the outcome of intrinsic competition. When *B. galactopus* had a 48 h head-start, they won almost 90% of the competitive

interactions (binomial test, P < 0.001; Figure 2). However, when *M. gemellus* oviposited first, *B. galactopus* experienced a reduced success rate (~ 55%) in competition (binomial test; P = 0.83; Figure 2).

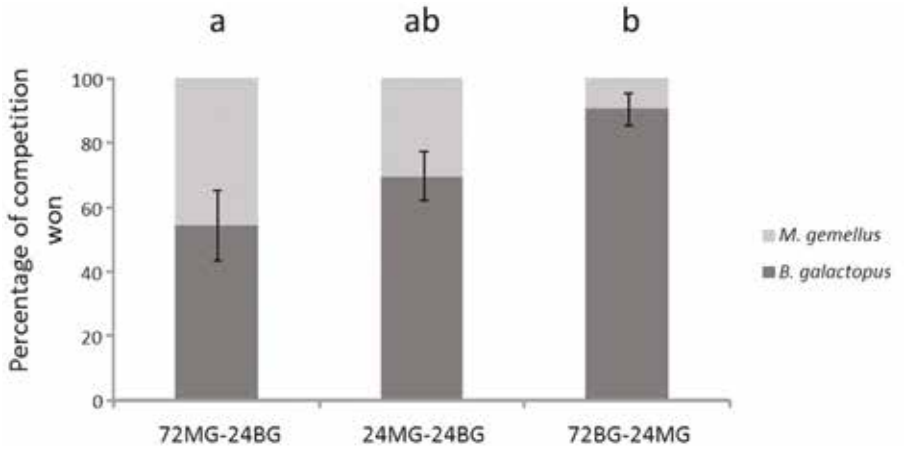

Figure 2. Percentage of *Mesochorus gemellus* or *Baryscapus galactopus* winning the intrinsic competetion. The three multiparasitism schemes were, 72MG-24BG: hyperparasitism by *M. gemellus* at 72 hours and *B. galactopus* at 24 hours before larval egression of *C. rubecula*; 24MG-24BG: hyperparasitism by *M. gemellus* and *B. galactopus* simultaneously at 24 h before larval egression of *C. rubecula*; 72BG-24MG: hyperparasitism by *B. galactopus* at 72 hours and by *M. gemellus* at 24 hours before larval egression of *C. rubecula*. Different letters above bars indicate differences (*Pearson's chi-squared tests*, P < 0.05).

Hyperparasitism success, in terms of the winning hyperparasitoid species, was affected by intrinsic competition. First, survival of *B. galactopus* was higher in *C. rubecula* (70% of the cases) in the absence of competition than when *B. galactopus* was competing with *M. gemellus* (less than 50% in 72MG-24BG and 24MG-72BG) (*Pearson's chi-squared test*, χ^2 = 26.893, df = 4, P < 0.001; Figure 3). Second, intrinsic competition also affected hyperparasitism success of *M. gemellus*, which experienced lower survival under competition with *B. galactopus* than when developing alone in a host (*Pearson's chi-squared test*, χ^2 = 24.321, df = 4, P < 0.001; Figure 3). The two time points for hyperparasitism (72 h or 24 h before *C. rubecula* larval egression) did not influence success rate of development for either hyperparasitoid species (Figure 3).

Mesochorus gemellus completed its development more rapidly than *B. galactopus* (Kaplan-Meier survival test; χ^2 = 92.647, df = 1, P < 0.001; Figure 4). In addition, we found that intrinsic competition did not affect the performance of *B. galactopus* in terms of egg-to-adult development time, clutch size, mean adult fresh body mass and sex ratio (Table 1). For *M. gemellus*, egg-to-adult development time but not mean adult fresh body mass was affected by different hyperparasitism treatments (Table 1). Development time of *M. gemellus* was longer when the wasps were involved in intrinsic competition with *B. galactopus* (e.g. 72MG-24BG) than in the absence of competition (72MG).

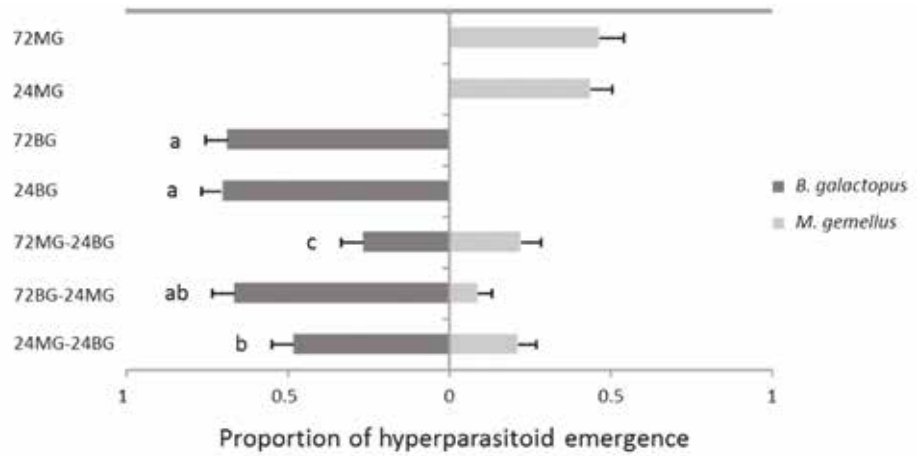

Figure 3. Proportions of hyperparasitoids emerged from single- or multi-hyperparasitism. 72MG & 24 MG: hyperparasitsm by *Mesochorus gemellus* at 72 h or 24 h before larval egression of *C. rubecula*, respectively; 72BG & 24BG: hyperparasitism by *Baryscapus galactopus* at 72 h or 24 h before larval egression of *C. rubecula*; 24MG-24BG: hyperparasitism by *M. gemellus* and *B. galactopus* simultaneously at 24 h before larval egression of *C. rubecula*; 72MG-24BG: hyperparasitism by *M. gemellus* at 72 hours and *B. galactopus* at 24 hours before larval egression of *C. rubecula*; 72BG-24MG: hyperparasitism by *B. galactopus* at 72 hours and by *M. gemellus* at 24 hours before larval egression of *C. rubecula*. Different letters beside bars indicate differences for *Baryscapus galactopus* (lower case letters) and *Mesochorus gemellus* (upper case letters), by pair-wise comparisons using Pearson's chi-squared tests, $P < 0.05$.

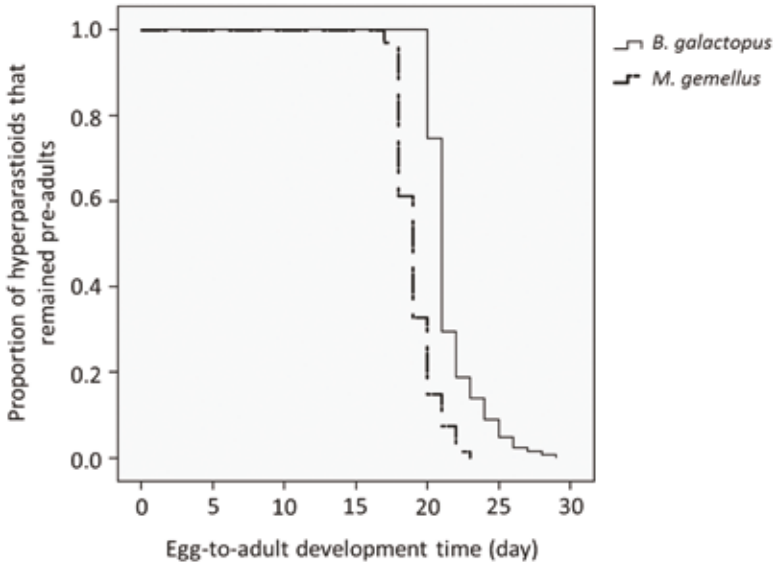

Figure 4. Comparison of egg-to-adult developmental time of *Mesochorus gemellus* and *Baryscapus galactopus*. The data were statistically analysed using the Kaplan-Meier survival test, $P < 0.001$.

Discussion

We examined a natural interaction involving a trophic chain with species over four trophic levels that are likely to interact in an insect community associated with *brassica*ceous plants across Eurasia. The results reveal that the gregarious primary hyperparasitoid *B. galactopus* had a competitive advantage over the solitary primary hyperparasitoid *M. gemellus*. However, the degree of superiority was to some extent context dependent, based on which species was the first to oviposit into larvae of their shared primary parasitoid host, *C. rubecula*. When *B. galactopus* had a temporal head start over *M. gemellus* in terms of the oviposition sequence, it won virtually all contests. However, when *M. gemellus* had a head start over *B. galactopus*, the competitive superiority of the latter species, although still evident, was less pronounced. The effect of competition on development time of the winning parasitoid was evident only in *M. gemellus,* whereas no other effects on fitness-related traits in either species were observed.

Intrinsic competition among parasitoid wasps has received considerable attention over the years, but thus far the vast majority of studies have been based on experiments with primary larval koinobiont endoparasitoids of lepidopteran larvae (Harvey *et al.* 2013). A general pattern that has emerged from this work is that the outcome of competition depends on the temporal interval between the first and second parasitism. For instance, when there is a time lag between the first and second oviposition event, the first parasitoid to oviposit generally outcompetes later arriving parasitoids (Tillman & Powell 1992; De Moraes *et al.* 1999; Wang *et al.* 2003; Harvey *et al.* 2009a; Sidney *et al.* 2010). One recent study demonstrated a solitary secondary hyperparasitoid, *Gelis agilis* in competition with another solitary secondary hyperparasitoid, *Lysiba nana,* in cocoons of their host, *Cotesia glomerata*. The species to attack first won most frequently when it had a 24-48 h head start. Remarkably, *G. agilis* became dominant when *L. nana* was offered a head start of more than 72h, revealing that *G. agilis* is also a tertiary hyperparasitoid of *L. nana*. However, the reverse was not true: *L. nana* rejected pre-pupae and pupae of *G. agilis* in cocoons of *C. glomerata*, revealing that it is specialized on primary parasitoid hosts only (Harvey *et al.* 2011b).

Here, we also found that time lag influences the outcome of intrinsic competition between the two primary hyperparasitoids, in which the first arriving hyperparasitoid species gained a competitive advantage over a second hyperparasitoid species that subsequently parasitized the host. As a superior competitor, *B. galactopus* won most contests in both 24MG-24BG and 72BG-24MG experimental setups. However, this competitive superiority was lost when *M. gemellus* had a head start in oviposition (72MG-24BG). One of the major advantages of being the first parasitoid to oviposit (mother) or hatch (her progeny) inside a host is that the parasitoid can manipulate various aspects of host growth and immunosuppression that facilitate their own

Table 1. Performance of hyperparasitoid *Baryscapus galactopus* and *Mesochorus gemellus* under intrinsic competition. Data for hyperparasitoid life history traits are given as mean (SE). For gregarious hyperarasitoid *B. galactopus*, SEs are calculated per host individuals. Significant differences in performance parameters among single and multi-hyperparasitism treatments are indicated in bold face .

Life history traits	Hyperparasitism schemes[x]							P-value (ANOVA)
	Single hyperparasitism				Multi-hyperparasitism			
	72MG (N = 45)	24MG (N = 57)	72BG (N = 45)	24BG (N = 57)	24MG-24BG (N = 56)	72MG-24BG (N = 45)	72BG-24MG (N = 45)	
Baryscapus galactopus								
Development time (day)	/	/	21.1 (0.21)	21.4 (0.26)	21.5 (0.44)	21.8 (0.73)	22.1 (0.38)	0.305
Clutch size	/	/	10.8 (1.31)	10.8 (1.16)	11.9 (1.41)	11.4 (2.11)	12.2 (1.36)	0.916
Fresh weight (µg)	/	/	165.2 (18.74)	171.1 (15.40)	174.1 (19.13)	195.7 (29.63)	144.5 (17.71)	0.599
Sex ratio[y]			66.3 (4.59)	74.2 (3.99)	75.0 (4.96)	74.1 (7.68)	62.0 (4.59)	0.202
Mesochorus gemellus								
Development time (day)	18.5 (0.25)[b]	19.5 (0.31)[a]	/	/	19.4 (0.36)[ab]	19.8 (0.44)[a]	18.5 (0.29)[ab]	**0.040**
Fresh weight (mg)	1.7 (0.08)	1.6 (0.09)	/	/	1.7 (0.11)	1.5 (0.12)	1.7 (0.19)	0.478

[x]: Hyperparasitsm schemes include: 72MG & 24 MG: hyperparasitsm by *Mesochorus gemellus* at 72 h or 24 h before emergence of *C. rubecula*, respectively; 72BG & 24BG: hyperparasitism by *Baryscapus galactopus* at 72 h or 24 h before emergence of *C. rubecula*, respectively; 24MG-24BG: hyperparasitism by *M. gemellus* and *B. galactopus* simultaneously at 24 h before emergence of *C. rubecula*; 72MG-24BG: hyperparasitism by *M. gemellus* at 72 hours and *B. galactopus* at 24 hours before emergence of *C. rubecula*; 72BG-24MG: hyperparasitism by *B. galactopus* at 72 hours and by *M. gemellus* at 24 hours before emergence of *C. rubecula*.

[y]: Percentage of females

development but which may be harmful to later-arriving competitors (Dahlman 1990; Godfray 1994; Strand & Pech 1995). A previous study suggested that *B. galactopus* larvae may release secretions or feed on specific host tissues and thus prevent further development of their host once it has egressed from the host (Harvey *et al.* 2012). In this way the development of other primary and/or secondary hyperparasitoids may be negatively affected.

In addition to the sequence of oviposition, the growth rate of immature parasitoids is another important factor that may affect the outcome of intrinsic competition. Selection may favor a reduction in the duration of embryonic and/or larval development that reduces the exposure of immature parasitoids to competitors, a process described as the 'slow-grow-high-mortality hypothesis' (Clancy & Price 1987; Benrey & Denno 1997). For competing hyperparasitoid larvae, a faster development rate results in a more rapid utilization of host resources and earlier pupation. In contrast with this argument, *B. galactopus* was a superior competitor to *M. gemellus* even though it requires a longer period to complete its development to adult eclosion. We have found that, when developing in fully-grown larvae of *C. rubecula*, *B. galactopus* attain their highest growth rate within 3-5 days of oviposition and fully consume their host within only six days of oviposition (unpublished data). Considering that *B. galactopus* generally requires 20-24 days to develop from egg to adult (Harvey *et al.* 2012; Zhu *et al.* 2014b), this reveals that they develop very rapidly as eggs/larvae but slowly as pupae. Thus far, we still lack knowledge on the duration of development for immature stages of *M. gemellus*. Possibly, *B. galactopus* may outcompete *M. gemellus* because it has a shorter egg or larval developmental time, thus exploiting host resources before *M. gemellus* larvae have begun to consume significant amounts of host tissues.

Although evidence indicates that solitary species are superior competitors in primary parasitoids (Laing & Corrigan 1987; Magdaraog *et al.* 2012; Poelman *et al.* 2014), our results show that a gregarious hyperparasitoid outcompeted its solitary rival. In primary parasitoids, solitary larvae are more mobile during the first-instar stage and have well-developed biting mandibles compared to larvae of gregarious parasitoids that allow the larvae of the solitary species to attack and kill competing larvae in the host (Fisher 1961, 1962; Harvey *et al.* 2013). These aggressive behaviours and mobility have been thought to be lost as a consequence of kin selection in the evolution of gregariousness (Godfray 1987; Ode & Rosenheim 1998; Boivin & van Baaren 2000; Pexton & Mayhew 2004). It has been suggested that gregarious larvae may still defend themselves against other species, although they do not actively seek out their competitors (van Nouhuys & Punju 2010). Furthermore, larvae of a solitary species may not be able to seek out and physically attack all gregarious larvae when they are present in a large number (van Nouhuys & Punju 2010; Harvey *et al.* 2013). In addition to inter-specific physical combat, host resource utilization is also an important

factor influencing the outcome of competition. Gregarious koinobionts may frequently experience scramble competition when the brood size exceeds an optimal load for a single host, resulting in a negative effect on per capita parasitoid fitness (Gu *et al.* 2003; Harvey *et al.* 2013). Despite scramble competition, gregarious endoparasitoids show intraspecific resource sharing as a common observed phenomenon. However, resource sharing is hardly found with solitary koinobionts. Our results suggest that early hatching gregarious larvae have advantages in competition by partitioning the limited host resources, reducing the potential for solitary larvae to acquire sufficient resources for development.

It has been frequently found that winner species involved in intrinsic competition may experience reduced fitness, such as body mass and an extended developmental time (Harvey *et al.* 2013; Poelman *et al.* 2014). Here, we found that the life-history traits (including development time, clutch size, sex ratio and fresh weight) of the winner were comparable to adult hyperparasitoids that were not involved in intrinsic competitions. This may indicate that the key to be competitive in interspecific intrinsic competition is monopolization of host resources. It is likely that *B. galactopus* are good at monopolizing limited host resources at early larval stages, especially when they have a head start. Therefore, young larvae of *M. gemellus* find themselves with insufficient resources for development, resulting in early mortality of the solitary larvae. When *M. gemellus* gained a head start, they may either kill or chemically suppress the egg or larvae of *B. galactopus*. In this way, the larvae of *M. gemellus* may efficiently kill the competitors without expending too much time and energy (Harvey *et al.* 2013). Once they allow hatching of *B. galactopus* eggs, they can hardly win the competition.

We have shown in the current study that the gregarious hyperparasitoid *B. galactopus* is a superior competitor in intrinsic competition with a solitary species *M. gemellus*, when feeding within their common host *C. rubecula*. This outcome is in contrast to most of the studies focusing on primary parasitoids, which suggest that solitary species outcompete gregarious species. Our results show that intrinsic competition may play an important role in determining the composition of hyperparasitoid communities. However, we know little of the extent to which these two hyperparasitoids compete for hosts in nature when including extrinsic forms of competition. Given that the adults of *M. gemellus* have much larger body size, they may obtain better dispersal abilities and be able to search for hosts over a much wider area than *B. galactopus*. This may lead to advantages in extrinsic competitions for *M. gemellus*. Furthermore, *Cotesia* species are generally hosting more hyperparasitoid species, including both primary and secondary hyperparasitoid (Harvey *et al.* 2009c; Poelman *et al.* 2012; Poelman *et al.* 2013). How these species interact in nature and what could be the outcome of intrinsic competitions between primary and secondary hyperparasitoids, still remains to be identified in future studies. Linking different life-history traits and outcome of

competitive interactions among hyperparasitoids, may yield further insights into host parasitoid food webs and the role of hyperparasitoids in natural and agroecosystems.

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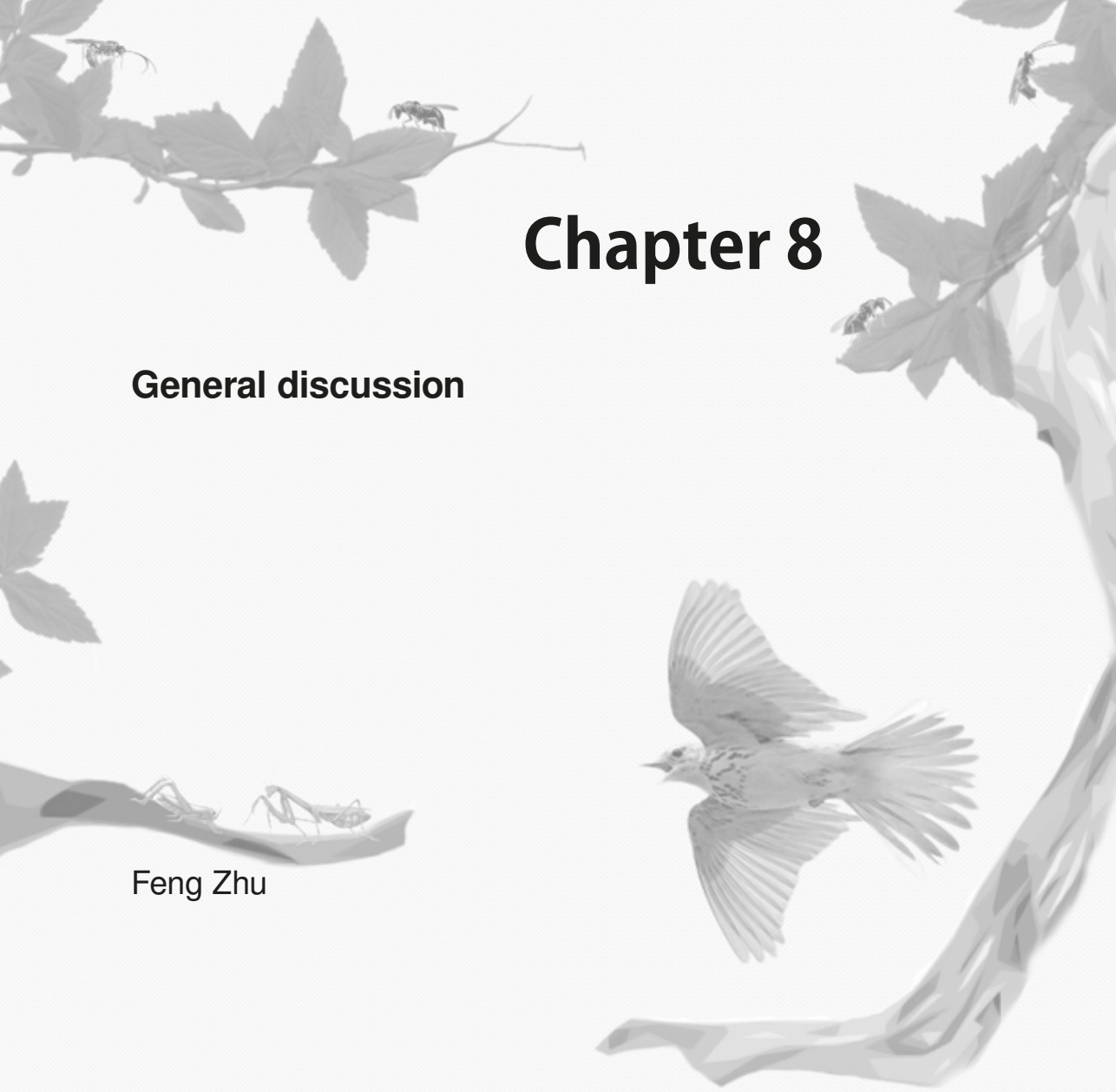
Chapter 8

General discussion



Feng Zhu

Introduction

The reciprocal interactions between plants and insects have a long evolutionary history (Schoonhoven *et al.* 2005). Over the past decades, substantial progress has been made in different insect-plant systems regarding insect-plant coevolution, plant direct and indirect defence against herbivores, as well as plant trait-mediated species interactions in multitrophic systems (Thompson 2005; Dicke 2009; Dicke & Baldwin 2010; Agrawal *et al.* 2012; Mithofer & Boland 2012; Bruce 2015). During their life-time, plants may be visited by many species of insects that include both harmful herbivores and beneficial natural enemies of herbivores. Plants exhibit changes in their traits (altered phenotypes) when interacting with one species, which may subsequently affect the performance and behaviour of other species. Thereby, plant traits mediate interactions among members of the plant associated community and affect the structure and dynamics of insect communities (Utsumi & Ohgushi 2008; Dicke & Baldwin 2010; Poelman *et al.* 2013). By realizing that plant-based food webs and complexity of direct and indirect interactions between species are diverse (Kaplan 2012), an increasing number of plant-associated organisms and environmental factors have been taken into consideration for designing study systems, which makes the study systems becoming more and more realistic in reflecting natural situations (Bezemer & van Dam 2005; Kogel *et al.* 2006; Poelman *et al.* 2008a; Poelman *et al.* 2011a; Kessler *et al.* 2013; Pineda *et al.* 2013; Stam *et al.* 2014; Li & Blande 2015; Weldegergis *et al.* 2015).

Plant-based food webs can be generally extended up to the fourth trophic level, that includes hyperparasitoids for example (Sullivan 1987; Sullivan & Volkl 1999; Harvey *et al.* 2003). In nature, hyperparasitoids actively attack parasitoids, the natural enemies of herbivore insects, resulting in a hyperparasitsm rate of up to 50 percent in parasitoid hosts (Poelman *et al.* 2012). Thus far, although a number of studies assessed life-history traits and host preference and performance of hyperparasitoids (Harvey *et al.* 2003; Buitenhuis *et al.* 2004; Harvey *et al.* 2012), little is known about the cues they use for host location or how primary parasitoids are involved in interactions between hyperparasitoids and host plant or host herbivore (Sullivan & Volkl 1999; Buitenhuis *et al.* 2005). The main aim of this thesis project was to investigate the involvement of volatile chemical cues in mediating interactions among species from four trophic levels in the *brassica*ceous plant-associated arthropod food web. Furthermore, I aimed to identify the direct and indirect effects of the presence of parasitoid larvae (as an example of herbivore-associated organisms) on herbivore hosts and their food plant, respectively.

In this chapter, I discuss the findings of this thesis in comparison with results yielded from recent studies in related research fields. This research program took an

ecogenomic approach (Dicke *et al.* 2004), combining transcriptomics, metabolomics, and insect behavioural assays to address the interactions between *Brassica oleracea* and its associated insect community. The objective of this discussion is to place the results of my thesis into the broader perspective of chemical and molecular ecology of direct and indirect species interactions.

Host location and ecology of hyperparasitoids

Hyperparasitoids are a group of highly evolved parasitic wasps at the fourth trophic level of the insect community that attack the natural enemies (parasitoids) of herbivorous insects (Sullivan 1987). They have a broad diversity and distribution in different plant-associated insect communities (Sullivan 1987; Tanaka *et al.* 2007; Poelman *et al.* 2012), and may affect terrestrial herbivorous arthropod populations and the structure of the arthropod community through their effects on parasitoids (Rosenheim 1998; Sullivan & Volkl 1999). Thus far, studies have focused on the preference and performance of hyperparasitoids (Harvey *et al.* 2003; Buitenhuis *et al.* 2004; Ashfaq *et al.* 2005; Harvey 2008; Harvey *et al.* 2012); however, little is known regarding their foraging behaviour or the cues used for locating their hosts. For an aphid hyperparasitoid, *Euneura augarus*, Volkl and Sullivan (2000) suggested that host-plant specific volatiles may provide the hyperparasitoids with information on the presence of host parasitoids. However, a study using four different aphid hyperparasitoid argued that olfactory cues may not be the essential cues for host searching by hyperparasitoid females (Buitenhuis *et al.* 2005).

In this PhD project, olfactory cues used during host searching by hyperparasitoids that are closely associated with *Brassica* plants and their specialist herbivore *Pieris* caterpillars were studied. Interestingly, our results show that hyperparasitoids associated with caterpillars, such as *Lysibia nana*, respond to HIPVs during foraging for hosts (Poelman *et al.* 2012) (Chapters 3-5). In addition, these hyperparasitoids exhibit the ability to discriminate between HIPVs induced by unparasitized caterpillars and caterpillars carrying their developing host larvae (Chapter 3 & 4). These findings were confirmed under both laboratory and field conditions. Not only do hyperparasitoids use HIPVs for host location, but also the body odours of the herbivore host may give away the presence of parasitoid larvae to the primary hyperparasitoid *Baryscapus galactopus* (Zhu *et al.* 2014b) (Chapter 6). Volatiles emitted by parasitized caterpillars showed higher attraction to *B. galactopus*, which allow the hyperparasitoid to distinguish unparasitized from parasitized caterpillars, thereby resulting in a faster first contact and longer mounting period on parasitized caterpillars (Chapter 6). These findings suggest that olfactory cues from different sources (plants and herbivore hosts) are used by hyperparasitoids during host searching.

Community-wide consequences of herbivore-induced plant responses

Plants have evolved specific induced responses to cope with attack by various herbivorous insects. Feeding guild of the herbivore, i.e. being a leaf chewer versus sap sucker, is one of the important aspects that affect herbivore-induced plant responses (Bidart-Bouzat & Kliebenstein 2011; Broekgaarden *et al.* 2011; Zhang *et al.* 2013). Moreover, food plant specialisation of the herbivore, i.e. being a generalist versus specialist, may also affect the nature of induced plant responses (Voelckel & Baldwin 2004; Bidart-Bouzat & Kliebenstein 2011; Ali & Agrawal 2012). These differentially induced responses to herbivory in plants consequently lead to a change in plant quality that may profoundly affect the performance of subsequent herbivores (Agrawal 2000; Kessler & Baldwin 2004; Poelman *et al.* 2011b). The induced changes in plant phenotype may also be perceived by other community members and affect their response to the induced plant, thereby affecting the structure of the plant-associated insect community (Broekgaarden *et al.* 2010; Utsumi 2011; Kaplan 2012; Poelman *et al.* 2012; Stam *et al.* 2014).

Some studies have demonstrated that herbivore-damaged plants repel herbivores searching for an oviposition site or negatively affect the performance of subsequently feeding herbivores (Bernasconi *et al.* 1998; Agrawal 2000; Kessler & Baldwin 2004; Zakir *et al.* 2013), whereas induced plant responses may also attract other herbivores that harm the plant. For example, a specialist herbivore on *Brassica*ceae plants, *Plutella xylostella,* prefers plants previously damaged by heterospecific herbivores for oviposition (Poelman *et al.* 2008a; Poelman *et al.* 2011b) (Chapter 5). Besides affecting the performance and host-selection behaviour of subsequently arriving herbivores, carnivores at the third trophic level may take advantage of herbivore-induced plant responses (e.g. emission of herbivore-induced plant volatiles [HIPVs]) for the location of their herbivorous hosts or prey (Vet & Dicke 1992; Dicke & Baldwin 2010; Kessler & Heil 2011). These HIPV-mediated indirect species interactions extend up to the fourth trophic level (Chapters 3-5). Besides the plant-associated insect community, herbivore-induced plant volatiles, as "public" cues, can also elicit behavioural changes in various other community members, occurring belowground or aboveground (Rasmann *et al.* 2005; Baldwin *et al.* 2006; Kost & Heil 2006; Runyon *et al.* 2006).

The relative importance of bottom-up (resource-based) and top-down (natural enemy-based) forces in shaping arthropod communities has long been debated (Rosenheim 1998; Ode 2006; Gripenberg & Roslin 2007). Several empirical researches indicate that plant quality (bottom-up effect) may affect the organisation of the community (Ode 2006; Bukovinszky *et al.* 2008; Kos *et al.* 2011; Santolamazza-Carbone *et al.* 2014). In *brassica*ceous plants, intraspecific variation in plant chemistry (glucosinolates and

HIPVs) profoundly affects plant resistance to herbivores and the plant's interactions with natural enemies of herbivores (Poelman *et al.* 2008b; Kos *et al.* 2011). However, evidence supporting trophic cascades (top-down effects) in plant-associated arthropod communities is lacking in comparison to those studies using aquatic ecosystems (Chase 2000). The possible reason is that natural enemies of herbivores are generally not top predators in the food web. In a conventional tri-trophic study system, it has been often ignored that natural enemies of herbivores at the third trophic level have an intermediate position in the complex food web. The potential top-down effect of hyperparasitoids on shaping the structure of the arthropod community has received little attention. The current conclusion is that both bottom-up and top-down effects play important roles in structuring arthropod community (Hunter & Price 1992; Rosenheim 1998). A better awareness of the ecological roles of organisms at the fourth trophic level and the community-wide consequences of herbivore-induced plant responses will definitely benefit further understanding of effects of bottom-up and top-down forces on population dynamics of plant-based terrestrial arthropod food webs.

Herbivore-associated organisms (HAOs)

Parasitoids affecting herbivore properties and plant responses to herbivory

The presence of parasitoid larvae inside a herbivore may affect herbivore properties directly. Firstly, my results demonstrated physiological changes in the herbivore that were induced by the presence of parasitoid larvae that developed in the herbivore. Volatiles emitted by parasitized caterpillars differed from those released by unparasitized *P. rapae* caterpillars, resulting in a differential attraction to primary hyperparasitoids (Chapter 6). Moreover, the presence of parasitoid larvae induces transcriptional changes in the host caterpillar, particularly in the labial salivary glands (Chapter 5). Besides inducing physiological changes, parasitoid larvae are able to induce behavioural changes in their herbivore hosts as well (Chapter 2). Although parasitoid larvae hardly make direct contact with host plants, our results indicate that they are able to indirectly affect plant responses to herbivory (Chapter 3 & 4). When parasitoid larvae are developing inside a herbivore, the parasitized *Pieris* caterpillars induced both metabolic changes (e.g. HIVPs) and transcriptional changes (defence-related genes) in *B. oleracea* plants (Poelman *et al.* 2011b; Poelman *et al.* 2012). This is likely caused by changes in the composition of herbivore regurgitant where major herbivore-associated elicitors have been identified (Poelman *et al.* 2011b; Bonaventure 2012). Here, herbivore hosts that carry parasitoid larvae are just an example of the many HAOs that may be present in herbivores.

HAO community

Apart from knowing that all higher organisms are featured by their biological complexity (McShea 1991), it should be realized that each higher organism has never been an anatomically independent individual (Gilbert *et al.* 2012). In terrestrial ecosystems, plants may benefit from microorganisms (such as endophytes) by gaining defensive properties against their herbivore attackers (Gange *et al.* 2012). Similarly, there is ample documentation for diverse organisms that live on or in herbivore hosts, both macro- and micro-organisms (Douglas 2015)(Chapter 2). Those macro-organisms, such as parasitic worms and parasitic insects that are associated with insect herbivores are mostly featured by their parasitic life history (Hughes *et al.* 2012). Moreover, herbivore-associated micro-organisms consist of numerous species of symbiotic bacteria or other environmentally acquired microbes (Moran *et al.* 2008; Hughes *et al.* 2012). Among insect symbiotic microbes, obligate symbionts are required for the survival of their host, whereas facultative symbionts are not essential for the survival of their hosts (Frago *et al.* 2012). Recent advanced genomics tools provide new avenues for the study of the HAO community and revealed that many insect species harbour diverse communities of microorganisms (Dillon & Dillon 2004; Moran *et al.* 2008; Hansen & Moran 2014). Although many of the HAOs confer a large impact on host development (Godfray 1994), nutritional utilization (Douglas 2009), and immune modulation (Hansen *et al.* 2012; Oliver *et al.* 2014), the role of HAOs in plant-insect interactions is still an emerging field.

HAOs affect plant-herbivore interactions

The significance of HAOs in plant-herbivore interactions was demonstrated by studies on obligate and facultative symbiotic microbes of sap-sucking insects. For instance, *Bemisia tabaci* whiteflies benefit from vectoring a begomovirus that can suppress the biosynthesis of major defence compounds in tobacco plants (Luan *et al.* 2013). Similarly, microbes in honeydew excreted by aphids may interrupt defence-related phytohormone accumulation in plants that become less resistant to aphids (Schwartzberg & Tumlinson 2014). Suppression of host plant defence by insect symbionts has also been illustrated for microbes present in oral secretions of Colorado potato beetles (Chung *et al.* 2013). Yet, little is known regarding the effects of HAOs residing in lepidopterans on interactions between plants and caterpillar hosts, especially the role of microbes in caterpillar oral secretion and their effect on plant responses to caterpillar feeding. Thus far, studies revealed relatively simple (limited number of species) bacterial communities in the midgut of lepidopteran larvae (Broderick *et al.* 2004; Robinson *et al.* 2010). Several studies have reported that selected antibiotics can successfully manipulate gut microbiota in a range of Lepidoptera (Broderick *et al.* 2009; Hernandez-Martinez *et al.* 2010; Robinson *et al.*

2010; Jakubowska *et al.* 2013). Due to its relative simplicity in associated microbe community and potential to be manipulated with antibiotics, lepidopteran larvae may become an ideal model for studying bacterial community dynamics (Robinson *et al.* 2010), as well as contributions of HAOs to plant-herbivore interactions.

Plants defend themselves against insect herbivory with a broad range of toxic secondary metabolites (Mithofer & Boland 2012). However, many herbivorous insects have evolved counter-adaptation strategies that enable them to feed on chemically defended plants without apparent negative effects (Heckel 2014). The specialist herbivore *Pieris rapae* has well adapted to *Brassica* plants that contain defensive compounds, i.e. glucosinolates. It has been found that the genome of *P. rapae* caterpillars contains genes encoding nitrile-specifier proteins (NSP) that detoxify glucosinolates (Wittstock *et al.* 2004). In addition to that, recent screening of the microbiome of *P. rapae's* midgut revealed the presence of *Enterobacter* and *Escherichia* bacteria (Robinson *et al.* 2010). These bacteria have been identified for properties of bio-tansformation of glucosinolates in food chemistry studies (Mullaney *et al.* 2013). Therefore, in parallel to NSP detoxification mechanisms, microbes residing in the caterpillar gut may also play a role in counter-adaptation to plant chemical defences.

HAOs affect multi-trophic interaction networks

HAOs have profound effects on the host, both behaviourally and physiologically, consequently resulting in extended phenotypes of their hosts. These extended phenotypes of herbivore hosts subsequently affect plant responses to herbivory and multitrophic interaction networks (Chapter 2). The altered plant phenotypes induced by parasitized herbivores affect oviposition preference of a subsequently colonizing herbivore, *Plutella xylostella* (Poelman *et al.* 2011b) (Chapter 5). Hyperparasitoids showed intriguing preferences towards the differences in HIPVs induced by parasitized or unparasitized caterpillars (Poelman *et al.* 2012) (Chapters 3 & 4). This is likely caused by changes in the composition of herbivore oral secretions where major herbivore-associated elicitors have been identified (Poelman *et al.* 2011b; Bonaventure 2012). It is clear that caterpillars regurgitate on the plant while feeding (Vadassery *et al.* 2012). Because caterpillar regurgitant is highly complex in composition, it is difficult to pinpoint what is the key elicitor involved and where is the origin of the elicitor. A previous study revealed that saliva secreted by labial salivary glands is closely associated with caterpillar feeding (Musser *et al.* 2006). Furthermore, it has been demonstrated that caterpillar saliva plays important roles in plant-insect interactions using an ablation technique for labial salivary glands (Musser *et al.* 2006). By using a similar ablation technique, our results indicated that caterpillar saliva plays an important role in induced plant responses to herbivory. Furthermore,

the two biological indicators, *P. xylostella* and *L. nana*, showed different oviposition preference and foraging behaviour, respectively, to plants induced by intact healthy or parasitized caterpillars or each of those caterpillars with salivary glands ablated. Finally, transcriptomic analysis revealed effects of parasitism on gene transcription in herbivore labial salivary glands (Chapter 5). Therefore, HAOs are able to directly affect plant traits and, via the extended phenotype of herbivore hosts indirectly affect plant responses to herbivory as well, thereby significantly influencing plant-mediated interaction webs.

Whether HIPVs benefit plant fitness

In response to herbivore attack, plants emit complex mixtures of volatile organic compounds that have been demonstrated to be attractive to natural enemies of herbivores and to enhance their foraging efficiency. Thereby, the emission of HIPVs has been considered to function as an indirect defence mechanism (Vet & Dicke 1992; Kessler & Baldwin 2001; Bruce *et al.* 2005; Schoonhoven *et al.* 2005; McCormick *et al.* 2012). It has been suggested to breed crop cultivars with enhanced production of HIPVs for better natural enemy recruitment (Kappers *et al.* 2010; Kappers *et al.* 2011). However, whether increased HIPV production really functions as defence should be tested by measuring plant fitness (Kessler & Heil 2011). Some recent studies have shown that herbivore-damaged plants may gain a fitness benefit from the recruitment of natural enemies as “bodyguard” (van Loon *et al.* 2000; Hoballah & Turlings 2001; Smallegange *et al.* 2008; Schuman *et al.* 2012a). However, one should also be aware that HIPVs as airborne signals make plants apparent to other attacking herbivores as well (Dicke 1986; Kalberer *et al.* 2001; Halitschke *et al.* 2008). Given that hyperparasitoids respond to HIVPs for host location and are “the enemy of the herbivore’s enemy” (Chapters 3 & 4), the indirect defensive function of HIPVs should be revisited with consideration of the effects on hyperparasitoids and colonizing herbivores on the defensive effects of plant volatile-mediated interactions with community members in different plant-herbivore systems.

Thus far, there is little information available to make a valid prediction on whether the attraction of hyperparasitoids by HIPVs may influence plant fitness. Our results indicate that cocoon clutches attached on plants that had been infested by caterpillars parasitized by gregarious parasitoids received higher hyperparasitism rates than those attached on plants damaged by unparasitized caterpillars (Chapter 3). However, food consumption of *Pieris* caterpillars is not stopped due to parasitism, and hyperparasitism generally takes place when caterpillar hosts have reached their final larval stage, or have already completed their larval stages (Harvey *et al.* 2012; Poelman *et al.* 2012; Zhu *et al.* 2014b). As a result, hyperparasitism may not significantly affect plant fitness. However, because plant volatiles mediate a complex

species interaction network involving a large diversity of plant-associated harmful and beneficial organism, the defensive properties of HIPVs should be eventually evaluated in a community-wide context.

Concluding remarks and future perspective

The outcomes of this research program contribute to our understanding of the complexity of species interactions in natural food webs. The structure of food webs may be significantly affected by competition among species at the same trophic level (Chapter 7), or direct and indirect interactions among organisms at different trophic levels. By addressing the chemical cues used by hyperparasitoids during host searching, organisms at the fourth trophic level were included in our study system, which have received very little attention in the past. It was demonstrated that hyperparasitoids use HIPVs as cues during host searching and that they are able to distinguish between plant volatiles induced by parasitized caterpillars and those induced by unparasitized caterpillars (Chapters 3 & 4). Using transcriptomic and metabolomic approaches, it was found that both herbivore identity and parasitism affect plant responses to insect herbivory (Chapter 4). Differential plant responses induced by parasitized caterpillars may be caused by parasitism-induced changes in the caterpillars' saliva secreted by labial salivary glands (Chapter 5). In addition to using HIPVs, volatiles emitted by the caterpillar body also indicate the presence of parasitoid hosts to hyperparasitoids (Chapter 6). Moreover, HAOs (as "hidden players") profoundly affect plant-herbivore interactions, as well as plant-mediated indirect trophic interaction networks (Chapters 2, 4 & 5). The natural enemies of herbivores used in biological control may themselves be attacked by hyperparasitoids and elicit effects on plant responses to herbivory that may result in subsequent herbivore colonisation. Therefore, the effects of plant breeding programs that aim to enhance HIPV production for better recruitment of natural enemies should be evaluated in a community context including organisms at the fourth trophic level.

In the past few decades, many studies have yielded exciting outcomes in fields such as multitrophic plant-insect interactions, chemical and molecular ecology, which greatly contribute to understand the complex interaction networks in ecosystems. To better mimic what is happening in reality, research has developed from addressing interactions between one plant and one herbivore to involving more natural combinations including key players at different trophic levels (Kessler & Halitschke 2007; Dicke & Baldwin 2010; Stam *et al.* 2014). Although we have gained much knowledge on relationships among individual entities, there is a need to emphasize that each individual macroorganism constitutes a complex community in itself (Gilbert

et al. 2012; Zhu *et al.* 2014a), such as the endophyte community residing in plants (Kogel *et al.* 2006; Gimenez *et al.* 2007). It should also be realized that there are many dynamic changes of associated organisms taking place within each individual while their interactions with other organisms are being studied (Moran *et al.* 2008).

The knowledge emerging from the current research project on HIPV-mediated plant-hyperparasitoid interactions, together with other studies of tripartite microbe-plant-insect interactions and insect-microbe symbiosis urges us to be aware that HAOs are important hidden players in plant-insect interactions (van de Mortel *et al.* 2012; Biere & Tack 2013). The studies of the effects of HAOs on plant responses to herbivory need to be extended to involve different classes of HAOs and compare their effects. Since lepidopteran larvae have been suggested as model for studying the herbivore-associated microbiome, it opens opportunities to study the functions of HAOs associated with Lepidoptera on plant responses to herbivory, herbivore nutrition utilization (detoxification of plant secondary metabolites), and herbivore defence against natural enemies. Taking these next steps using integrated approaches, combining metagenomics, transcriptomics, proteomics and insect behavioural assays, will make the studies of plant-insect interactions more complex but also more fascinating, resulting in exciting developments in this field.

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Summary

How direct and indirect species interactions shape diversity and evolution of individual species or community composition is a central issue in ecology. In terrestrial ecosystems, plants are among the most important components and inhabit a large diversity of landscapes. As primary producers in food webs, plants are challenged by various herbivorous organisms. Among the herbivorous animals on the planet, insects are the most diverse group and have a long evolutionary history (about 350 million years) with their host plants. Plants evolved a suite of direct and indirect defence mechanisms to cope with insect attack, and significantly affect the structure of plant-associated insect communities. Such communities generally include carnivorous insects from the third trophic level and even from the fourth trophic level. It has been shown that parasitoids (at the 3rd trophic level) respond to herbivore-induced plant volatiles during localising of their herbivore hosts. However, less is known about the foraging cues used by hyperparasitoids (at the 4th trophic level) that develop in or on parasitoids. Hyperparasitoids have been considered as a threat to parasitoids that have potential value in biological control programs. There is a need for understanding of the cues used by hyperparasitoids in their foraging decisions.

The aim of this thesis was to investigate the cues that are used by hyperparasitoids in host location. In addition, we addressed the role of herbivore-associated organisms (HAOs) in plant-mediated indirect species interactions. In chapter 1 of this thesis, the four-trophic-level study system is introduced. The wild population “Kimmeridge”of *Brassica oleracea* plants hosts specialist herbivores, such as *Pieris rapae* and *P. brassicae*. *Cotesia glomerata* and *C. rubecula* are the natural enemies of *Pieris* caterpillars and spend their whole larval stages in the herbivore host. The *Cotesia* species may further be parasitized by a suite of primary hyperparasitoids (that attack parasitoid larvae) or secondary hyperparasitoids (that attack parasitoid pupae).

Host location by hyperparasitoids

In chapter 3, the responses of the natural secondary hyperparasitoid community to herbivore-induced plants were studied in field experiments. Under field conditions, plants were induced by unparasitized or parasitized *P. rapae* caterpillars. The parasitoid cocoons were subsequently attached to different herbivore-induced plants and exposed to hyperparasitoids. Cocoons attached to herbivore-damaged plants received higher hyperparasitism rates than those attached to undamaged plants. Interestingly, highest hyperarasitism rates were found in cocoons attached to plants damaged by caterpillars parasitized by the gregarious parasitoid *C. glomerata*, indicating that hyperparasitoids are able to distinguish plants damaged by caterpillars carrying larvae of different parasitoid species. Together with previous Y-tube

olfactometer assays, it is confirmed that the hyperparasitoid *L. nana* uses HIPVs as cues during host searching. Moreover, the presence of parasitoids indirectly affects plant-hyperparasitoid interactions.

In nature, a single parasitoid species may attack different herbivore species. Therefore, whether herbivore identity (*P. rapae* or *P. brassicae*) affects foraging preferences of hyperparasitoids was further investigated in Chapter 4, using an ecogenomic approach that combines insect behavioural assays with plant metabolomic and transcriptomic analyses. The herbivore identity of parasitized caterpillars did not influence the hyperparasitoid *L. nana's* preferences for HIPVs under both laboratory and field conditions, although it did affect plant transcriptional and metabolomic responses to herbivory. Compared to parasitism, herbivore identity plays a minor role in HIPV-mediated plant-hyperparasitoid interactions.

Apart from plant volatiles, a broad range of infochemicals are present in nature, which are may be used by hyperparasitoids for host location. In Chapter 6, I addressed whether volatiles emitted by herbivores themselves can be used by the primary hyperparasitoid *Baryscapus galactopus* for location of their inconspicuous hosts developing in the caterpillar. Furthermore, volatiles from the headspace of unparasitized and parasitized herbivores were collected to study whether parasitism affects body odours of herbivore hosts. Interestingly, *B. galactopus* responded to volatiles released by *P. rapae* caterpillars and can distinguish between body odours of unparasitized and parasitized herbivore hosts. The primary hyperparasitoids were faster in making first contact with parasitized caterpillars and spent longer mounting time on these hosts. Analysis of the headspace of caterpillars revealed that parasitoid larvae affect the physiology of their herbivore host, resulting in altered body odours of the caterpillar. Therefore, hyperparasitoids are able to use chemical cues that have different origins for host searching: plant and herbivore odours.

Herbivore-associated organisms

Similar to other higher organism, there are diverse micro-organisms (viruses, bacteria, fungi) and macro-organisms (parasitic worms or parasitic wasps) living in or on herbivorous insects. These herbivore-associated organisms (HAOs) may profoundly affect plant direct and indirect responses to herbivory. In Chapter 2, the examples regarding behavioural and physiological manipulations of herbivore hosts by HAOs are discussed. Some HAOs can modulate plant defensive responses to their herbivore host through direct contact with plant tissues. Whereas some other HAOs indirectly affect plant responses to herbivory via manipulating host feeding behaviours and physiological status. As "hidden players", HAOs may also drive plant-insect coevolution, as well as shape the structure of the insect community. Particularly, it has been shown that the presence of parasitoid larvae inside herbivore

hosts causes differentially expressed defence-related genes in plants. The altered plant traits further affect ovipostion preferences of the diamondback moth *Plutella xylostella* and foraging behaviours of the hyperparastioid *L. nana*. It was suggested that the altered plant phenotype induced by parasitized caterpillars was due to parasitism-induced changes in composition of caterpillar oral secretions, where several herbivore-associated elicitors (HAEs) were identified. Since caterpillar oral secretions are complex mixtures of substances with different origins, caterpillar labial saliva was studied for its role in plant-insect interactions in Chapter 5. Using an ablation technique for labial salivary glands, the secretion of labial saliva can be completely eliminated. The results showed that *P. xylostella* and *L. nana* cannot distinguish between plants induced by ablated unparasitized or parasitized caterpillars and respond to plants induced by ablated caterpillars similarly as to undamaged control plants. Plant volatiles induced by ablated or mock-treated caterpillars showed quantitative differences. Moreover, transcripts of genes encoding the herbivore-associated elicitors β-glucosidase and glucose oxidase were differentially regulated in salivary glands of parasitized caterpillars compared to unparasitized caterpillars. Therefore, the extended phenotype of parasitoid larvae that are expressed in changes in the saliva of their herbivorous host strongly influence plant trait-mediated indirect species interactions.

Intrinsic competition between primary hyperparasitoids

When different hyperparasitoids use the same parasitoid host, competitive interactions occur. For primary parasitoids, it has been suggested that solitary species are superior to gregarious species in intrinsic competition because of their aggressive nature. In Chapter 7, the intrinsic competition between two primary hyperparasitoids, *B. galactopus* and *Mesochorus gemellus*, was investigated. Remarkably, in contrast to what has been reported in the literature for primary parasitoids, the results of this study showed that the gregarious hyperparasitoid *B. galactopus* had a competitive advantage over the solitary species *M. gemellus*.

The outcomes of this thesis contribute to our understanding of the roles of infochemicals in foraging decisions of hyperparasitoids. The ecological roles of plant volatiles still require further investigations in a community-wide context. Although parasitoids may affect population dynamics of herbivorous insects, their presence in herbivores indirectly influences plant phenotypes and thereby result in altered trait-mediated indirect interaction networks that attract the hyperparasitoid enemies of beneficial third trophic level parasitoids.

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September, 2015
Feng Zhu

Curriculum Vitae

Feng Zhu was born on the 27th of May, 1981 in Tianjin, China. He finished a two-year educational programme in Horticulture at Tianjin Agriculture College in 2001. In the same year, he started working at Tianjin Speedling Co., Ltd, as manager of seedling production of vegetables and ornamental plants. In 2005, he started his Bachelor education in Horticulture and Agri-bussiness at HAS University of Applied Sciences in Den Bosch, the Netherlands. He conducted his BSc thesis at the Horticulture and Product Physiology Group of Wageningen University. Thereafter, he enrolled in the Master programme at Wageningen University with specialization in Genetic Resources and Plant Breeding, in 2008. He carried out a major MSc thesis on the topic of resistance breeding in tomato against *Clavibacter* at the Laboratory of Plant Breeding of Wageningen University under the supervision of Dr. Sjaak van Heusden and a minor thesis about using (meta)genomic methods to identify antimicrobial compounds at the Laboratory of Phytopathology of Wageningen University under the supervision of Dr. Menno van der Voort and Prof. dr. Jos Raaijmakers. From 2011, he started his PhD project on infochemical-mediated species interactions in food webs up to the fourth trophic level at the Laboratory of Entomology of Wageningen University, under the supervision of promoter Prof. dr. Marcel Dicke and co-promoter Dr. Erik Poelman. In this thesis, the results obtained during his PhD period are presented.

Publication list

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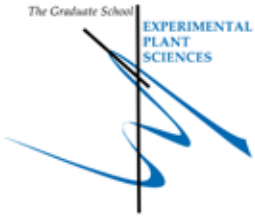
Education Statement of the Graduate School Experimental Plant Sciences



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1) Start-up phase	*date*
▸ **First presentation of your project**	
Ecogeomics: indirect plant-mediated interactions in food webs up to the fourth trophic level	Jun 07, 2011
▸ **Writing or rewriting a project proposal**	
▸ **Writing a review or book chapter**	
Insect herbivore-associated organisms affect plant responses to herbivory: New Phytologist, Volume 204, Issue 2, pages 315–321, October 2014	Oct 2014
▸ **MSc courses**	
Insect-Plant interactions (ENT-50806)	May-Jun 2011
▸ **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
▸ **EPS PhD student days**	
EPS PhD student day, Wageningen University	May 20, 2011
EPS PhD student day, University of Amsterdam	Nov 30, 2012
▸ **EPS theme symposia**	
Theme 2 'Interactions with plants and biotic agents', University of Amsterdam	Feb 03, 2011
Theme 2 'Interactions with plants and biotic agents', Wageningen University	Feb 10, 2012
Theme 2 'Interactions with plants and biotic agents', Utrecht Univeristy	Jan 24, 2013
Theme 2 'Interactions with plants and biotic agents', University of Amsterdam	Feb 25, 2014
▸ **NWO Lunteren days and other National Platforms**	
Netherlands Annual Ecology Meeting (NAEM) 2012	Feb 05-06, 2012
NWO-ALW Plant Sciences meeting, Lunteren	Apr 02-03, 2012
Netherlands Annual Ecology Meeting (NAEM) 2013	Feb 07-08, 2013
NWO-ALW Plant Sciences meeting, Lunteren	Apr 22-23, 2013
Netherlands Annual Ecology Meeting (NAEM) 2014	Feb 11-12, 2014
NWO-ALW Plant Sciences meeting, Lunteren	Apr 14-15, 2014
NWO-ALW Plant Sciences meeting, Lunteren	Apr 13-14, 2015

▸ **Seminars (series), workshops and symposia**	2011-2014
Plant Sciences Seminars	2011-2014
Wageningen Evolution and Ecology Seminar Series (WEES)	Jun 16, 2011
4th National Ecogenomics Day, University of Amsterdam	Jun 07, 2011
Symposium YELREM	May 30, 2012
Symposium YELREM	May 17, 2013
Symposium YELREM	May 21, 2014
Symposium YELREM	Nov 23, 2011
6th Workshop Plant-Insect Interactions, University of Amsterdam	Nov 28, 2012
7th Workshop Plant-Insect Interactions, Leiden University	Sep 24, 2013
8th Workshop Plant-Insect Interactions, Wageningen Univeristy	Nov 03, 2014
9th Workshop Plant-Insect Interactions, Utrecht Univeristy	Dec 16, 2011
23rd Entomologendag NEV	Dec 14, 2012
24th Entomologendag NEV	Dec 13, 2013
25th Entomologendag NEV	Nov 23, 2011
▸ **Seminar plus**	
▸ **International symposia and congresses**	
14th International Symposium on Insect-Plant Interactions, Wageningen, NL	Aug 13-18, 2011
15th International Symposium on Insect-Plant Relationships, Neuchâtel, Switzerland	Aug 17-22, 2014
Gordon Research Seminar (GRS) on Plant Volatiles, Ventura, USA	Jan 25-26, 2014
Gordon Research Conference(GRC) on Plant Volatiles, Ventura, USA	Jan 26-31, 2014
▸ **Presentations**	
NAEM, Lunteren (Poster)	Feb 08, 2013
GRS / GRC on Plant Volatiles, Ventura (Poster)	Jan 29, 2014
8th Workshop Plant-Insect Interactions, Wageningen (Talk)	Sep 24, 2013
25th Entomologendag NEV, Ede (Talk)	Dec 13, 2013
NAEM, Lunteren (Talk)	Feb 11, 2014
Theme 2 'Interactions with plants and biotic agents', Amsterdam (Talk)	Feb 25, 2014
15th International Symposium on Insect-Plant Relationships, Neuchâtel (Talk)	Aug 19, 2014
NWO-ALW meeting ‘Experimental Plant Sciences’, Lunteren (Talk)	Apr 14, 2015
▸ **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Jan 05, 2015
▸ **Excursions**	
PhD Trip, Switzerland	Oct 27-Nov 02, 2013
Subtotal Scientific Exposure	*28.1 credits**

3) In-Depth Studies	*date*
▸ **EPS courses or other PhD courses**	
PhD Summer School 'Environmental Signaling'	Aug 22-24, 2011
Bioinformatics: A User's Approach	Aug 17-21, 2012
▸ **Journal club**	
PhD lunch meeting Entomology	2011-2014
Insect-Plant Interactions, Laboratory of Entomology, WU	2011-2014
▸ **Individual research training**	
Subtotal In-Depth Studies	*7.5 credits**

4) Personal development	*date*
▸ **Skill training courses**	
Competence Assessment	Jan 24-Mar 01, 2012
Scientific Writing	May 08-Jun27, 2013
Project and Time Management	Nov 06-Dec 18, 2014
▸ **Organisation of PhD students day, course or conference**	
▸ **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*3.6 credits**

TOTAL NUMBER OF CREDIT POINTS*	**50.6**

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

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

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