Linking variation in plant defence to biodiversity at higher trophic levels: a multidisciplinary approach

Erik H. Poelman

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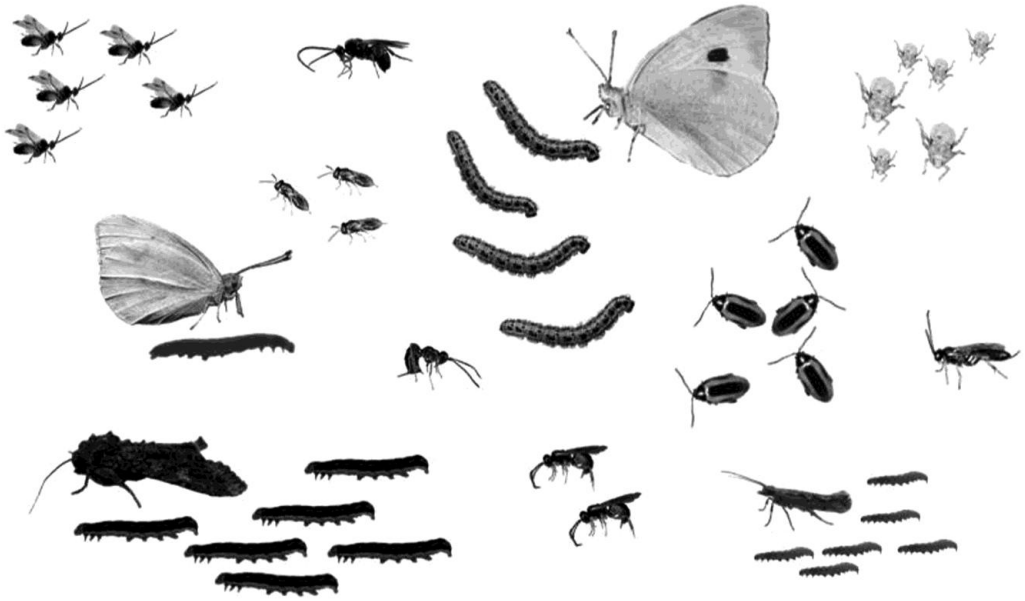
Abstract

Central issue in ecology are to identify the driving forces behind community diversity and the ways in which this biodiversity is maintained. Plant-insect associations form a significant part of the earth's biodiversity and are widely studied for their reciprocal interactions. The aim of this thesis was to identify the role of intraspecific variation in plant traits in affecting the diversity and composition of the plant-associated insect community. Cultivars of White Cabbage, *Brassica oleracea,* that differ in a set of defensive traits were used as a model system in conjunction with their insect community. The system was studied using a multidisciplinary approach that included targeted gene expression and chemical analyses of plant responses to insects as well as an ecological approach on the effect of plant defensive characteristics on insect communities. This project includes laboratory and field experiments.

The investigated *B. oleracea* cultivars differed widely in resistance to herbivorous insects, although the concentration of glucosinolates, secondary metabolites characteristic for Brassicaceae, did not correlate with cultivar resistance to herbivores. Herbivore-susceptible cultivars harboured higher abundance and diversity of herbivores compared to herbivore-resistant cultivars in the field. Both specialist and generalist herbivores of different feeding guilds covaried in higher abundance on herbivore-susceptible cultivars that characteristically had low concentrations of glucosinolates with a shorter side chain in their molecule structure.

Induction of the *B. oleracea* cultivars by *Pieris rapae* herbivory resulted in differential induction of chemical plant defences such as indolyl-glucosinolates and volatile organic compounds such as methyl salicylate. The different composition of the volatile blend emitted by the cultivars resulted in differential attraction of parasitoids to cultivars in an olfactometer assay. Cultivars showing higher attraction in this assay harboured a higher proportion of parasitized caterpillars in the field. The differences in indirect resistance among accessions had a two-fold effect on abundance and diversity of hyperparasitoids that parasitize on cocoons of the primary parasitoids. First, the larger number of cocoons sustained a larger number of hyperparasitoids and second the hyperparasitoids were also attracted to the plant accession that was most attractive to primary parasitoids. The full set of *P. rapae*-induced plant responses also affected the herbivore community. Plants that were previously damaged by *P. rapae* had different gene transcription responses to secondary herbivory than when these herbivore species were feeding as primary herbivore on a plant. The subsequently feeding herbivore species were all impeded in their performance, but had contrasting host plant preference for induced plants. Generalist herbivores avoided plants that were previously damaged by *P. rapae*, whereas specialist herbivores preferred induced plants. Thereby, early-season herbivory by *P. rapae* resulted in higher herbivore abundance and species richness compared to plants that had not experienced early-season herbivory in the field. Plant responses to early-season herbivory resulted in induced resistance to generalists, but in induced susceptibility to specialist herbivores of different feeding guilds.

In conclusion, the set of constitutive as well as inducible direct and indirect defensive traits of a plant affects the composition of the plant-associated insect community. The insect community as a whole should, therefore, be considered in studies of reciprocal selection between insects and defensive traits of plants.



Chapter 1

Consequences of variation in plant defence for biodiversity at higher trophic levels

Erik H. Poelman, Joop J.A. van Loon & Marcel Dicke

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Abstract

Plants are the basic food resource of complex communities. The organisms forming these communities in turn exert selection pressures on plant traits. Antagonistic species such as insect herbivores impose selection on plants to defend themselves against these attackers. Although selection on plant defence traits has typically been studied for pairwise plant-attacker interactions, other community members are unavoidably affected by these traits as well. A plant trait may e.g. affect parasitoids and predators feeding on the herbivore or other herbivores may be affected through induced changes in plant defence. Consequently, defensive plant traits structure the diversity and composition of the community associated with the plant and communities as a whole also feed back to selection on plant traits. Here, we review recent developments in the understanding of plant defence traits structuring insect communities and discuss how molecular mechanisms may drive community-wide effects.

Key words: plant defence, induced response, indirect defence, biodiversity

Plant traits and biodiversity

Central issues in ecology are to identify the driving forces behind community diversity and the ways in which this biodiversity is maintained. The ca. 300,000 different species of plants and 3 to 6 million species of insects, half of which are herbivorous, are a significant part of the Earth's total recorded biodiversity. Among the most common interactions occurring in ecosystems are plant-insect interactions. Studies of plant-insect associations have played an important role in understanding ecological and evolutionary processes that underlie community biodiversity (Whitham et al. 2006).

Recent studies have provided ample information on the molecular basis and ecology of plant defences against insects and pathogens. Constitutive defences of plants differentially affect various insect herbivores. These defences not only affect the performance of an attacker but may also influence the behaviour of the attacker, for example its host-plant selection behaviour (Schoonhoven et al. 2005). Upon damage by pathogens or herbivores, plants also respond to their attacker (Schaller 2008). Induced defence is observed as alterations in a set of traits that lead to a reduced effect of the attacker on plant fitness. Many of these induced plant defences have been studied in detail at both the molecular level and at the level of physiological consequences for insect performance, which revealed that plants are fine-tuning their responses specifically to their attackers (Heidel and Baldwin 2004; de Vos et al. 2005; Zarate et al. 2007; Pieterse and Dicke 2007). This research field recently addressed the question of how responses at the molecular and phytochemical level to initial attackers interact with responses to secondary attack or simultaneous attack by other organisms (Paul et al. 2000; Voelckel and Baldwin 2004; Bezemer and van Dam 2005; Viswanathan et al. 2007; Poelman et al. 2008b). It is now widely recognized that herbivore species may interact via induced plant responses even when they are not simultaneously present on the plant (Faeth 1986; Denno et al. 1995, 2000; Kaplan and Denno 2007). Plant responses induced by one species may affect the performance and also the behaviour of other species.

Glossary

Biodiversity: The number of species and their individual abundance in a given environment

Constitutive plant defence: defence of a plant that is expressed independent of attack

Community: the assembly of species in a particular area

Community-wide effect: an effect on many individual species constituting a community

Induced plant defence: the set of changes in a plant in response to an attack that impedes on plant fitness, which results in a reduced plant fitness loss inflicted by the attacking organism

Induced plant response: the set of changes, such as morphological and phytochemical, in a plant in response to an abiotic or biotic event impacting on the plant

Trophic cascade: an organism at one level of the food chain influences the abundance of the organisms at subsequent levels of the food chain

Trophic level: level in a food chain

Community ecologists have recently moved from a plant community perspective to a focus on intraspecific genetic variation in plants and its consequences for biodiversity at higher trophic levels (Dungey et al. 2000; Hochwender and Fritz 2004; Wimp et al. 2005; Bukovinszky et al. 2008). It appears that genotypic effects can be even stronger than envi-

Box 1. Community complexity

Plants form the basis of complex communities and thereby interact with a large number of attackers (Figure I). Insects contribute a significant part of plant attackers and a large part of the entire community associated with a plant. The diversity of attackers has driven the evolution of a broad scale of defence mechanisms in which plants defend themselves directly or indirectly against their attackers. Direct defences include plant morphology and chemistry that directly affect the attacker, whereas indirect defences promote the effectiveness of natural enemies of the plant attacker. Herbivorous insects are attacking plants above- as well as belowground and differ widely in their feeding mode. Some herbivores such as caterpillars are leaf chewers; others such as thrips are cell content feeders whereas aphids feed on phloem sap. Furthermore, within each of these feeding guilds herbivores differ in sensitivity to morphological or phytochemical defences, often depending on their host plant range. Generalist herbivores that have a wide host plant range are less well adapted to chemical defences that are specific for a host plant family. In contrast, specialist herbivores have adapted to these phytochemicals and often utilise the compounds in host plant recognition (Jaenike 1990, Schoonhoven et al. 2005). The presence of each of the herbivore species and their natural enemies varies geographically as well as in time and thereby exerts dynamic selection pressures on plant defences. Defence traits have been shown to vary considerably among plant species as well as within plant species with age, plant part, environmental conditions and, most importantly, plant genotype. Consequently, genotypes within a plant species interact differently with the community of attackers and harbour different insect communities.

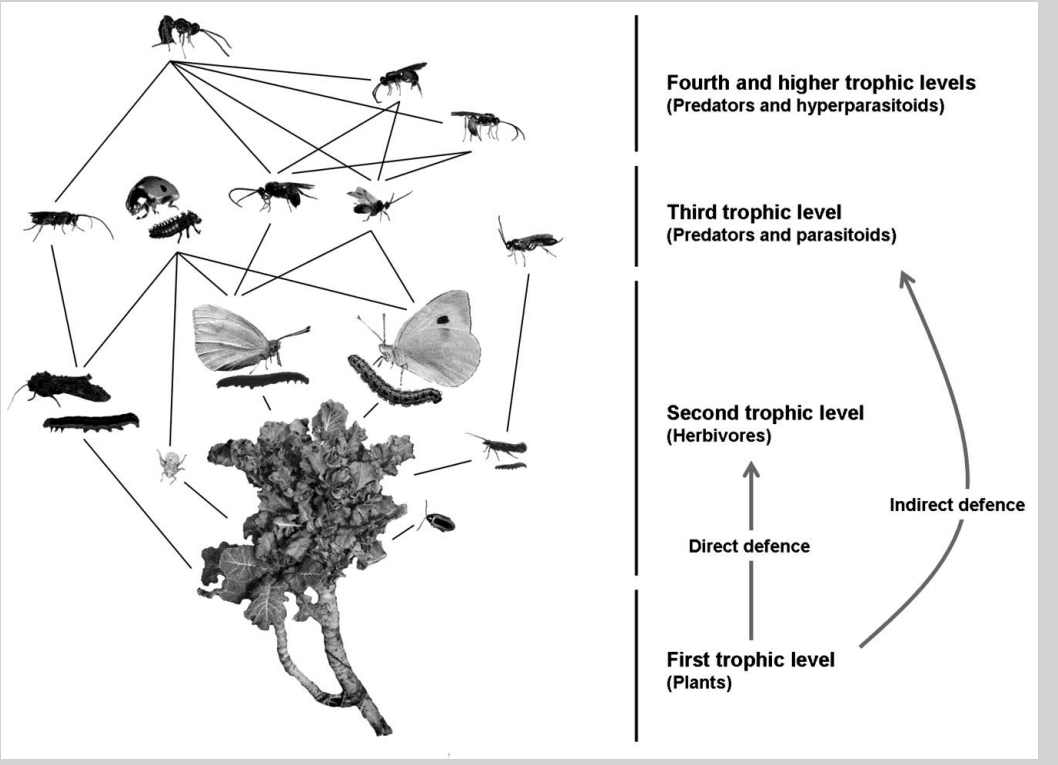

Figure I. *Brassica oleracea* plants harbour a community of herbivores and their natural enemies that form a complex of interacting associations. Plant defence traits directly or indirectly interact with each of these organisms and thereby affect the composition of the insect community. Furthermore, direct and indirect plant defences may have cascading effects over trophic levels.

ronmental effects in shaping insect herbivore communities (Johnson and Agrawal 2005; Bangert et al. 2006a) and genotypic effects have been shown to extend to the third trophic level and beyond (Gruner and Taylor 2006; Bailey et al. 2006; Bukovinszky et al. 2008). As a result of the recent expansion of their interests, the research fields of molecular biology and community ecology have transgressed each other's boundaries and established that variation in molecular mechanisms underlying plant defences structure biodiversity of higher trophic levels (Whitham et al. 2006, Zheng and Dicke 2008). Here, we review the recent literature on the interplay between molecular plant defence mechanisms and insect biodiversity. We will address: 1) how constitutive direct plant defences as well as inducible direct and indirect defences structure the associated insect community on the plant 2) whether plant defences affect groups of insect species that differ in host plant range similarly or differently 3) how community-wide effects of plant traits in their turn feed back to selection on plant traits.

Constitutive plant defences

Heritable plant traits are now widely recognized to affect the composition of an associated community (Whitham et al. 2006), and include constitutively expressed morphological (Johnson and Agrawal 2005) and phytochemical traits (Dungey et al. 2000; Bailey et al. 2006; Bangert et al. 2006b; Poelman et al. 2008a). Variation in constitutive defences directly affects members of the second trophic level, i.e. herbivores that are in close reciprocal interaction with plants, as seems intuitively logical. For example, the number of herbivore species that are found to be associated with natural hybrids of two plant species exceeds those on each of the parental species and typically consists of added subsets or even the whole community of both parental species (Dungey et al. 2000; Hochwender and Fritz 2004; Bangert et al. 2006b). The genetically based intermediate leaf shape as well as intermediate chemical composition of hybrids determines an intermediate herbivore community (Whitham et al. 1999; Dungey et al. 2000; Wimp et al. 2005; Bangert et al. 2006b). Within plant species, genotypes differ widely in phytochemistry and morphology and, consequently, also in herbivore abundance (Maddox and Root 1990). In brassicaceous plants, genotypes differ in the foliar composition of their characteristic secondary metabolites, i.e. glucosinolates (Mithen et al. 1995; van Leur et al. 2008; Gols et al. 2008b). The concentration of a single compound as well as the qualitative differences in the mixture of compounds is well known to differentially affect performance and preference for host plant genotype in different herbivore species (van Leur et al. 2008; Gols et al. 2008b). Phytochemical traits of genotypes, such as the composition of the secondary metabolites, consequently determine genotype-associated biodiversity of the herbivore community (Fritz and Price 1988; Maddox and Root 1990; Kessler and Baldwin 2004; Poelman et al. 2008a). Studies of genetically modified plants have unambiguously identified the genetic basis of plant resistance traits that influence herbivore abundance and community composition. Wild tobacco plants in which a key gene of the jasmonic acid (JA) pathway, that triggers the primary defence mechanism against leaf chewing herbivores, had been knocked out, harboured larger numbers of herbivores and were even colonized by a species that was never recorded on control plants in the field (Kessler et al. 2004).

Effects on the abundance of second trophic level organisms may directly or indirectly translate to differences in community composition of higher trophic levels (Bukovinszky et al. 2008). With different distributions of herbivores over plants, the availability of niches for organisms at higher trophic levels varies per plant. Thereby, abundance and diversity of the third trophic level is indirectly affected by heritable resistance traits affecting herbivores. These effects do not only shape the insect community of preda-

Box 2. Induced plant defence

Maintenance of constitutively expressed defences may be costly when herbivores are absent. Plants that mobilize some or all of their defences upon attack may benefit from not paying the costs of defence when herbivores are absent and can accurately pinpoint their defence responses to the type of attacker. Mobilization of plant defences is termed induced defence and can include changes in plant morphology, phytochemistry, production of extrafloral nectar and production of volatile organic compounds. The latter can be used by natural enemies in location of their herbivorous hosts and is termed induced indirect defence when these natural enemies effectively reduce the impact of the plant attacker on plant fitness. Members of different attacker guilds have been found to elicit profound differences in plant transcriptional responses including genes that encode defence traits (de Vos et al. 2005). The plant hormones jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) play a key role in the signalling of plant defences. Piercing-sucking insects, such as aphids, are known to elicit plant defence responses regulated by SA, whereas leaf chewing insects, such as caterpillars, elicit responses regulated by JA (Heidel and Baldwin 2004; de Vos et al. 2005; Zarate et al. 2007). Even within feeding guilds, different herbivore species elicit different responses in the plant (Voelckel and Baldwin 2004a). Upon sequential or simultaneous attack by different herbivore species, which is common in nature, plants need to tune their defences to a set of attackers above- as well as belowgound. Molecular responses may limit plants in accurately responding to attackers that demand different defences. For example, SA- and JA pathways have been found to act antagonistically by inhibiting the response to a subsequent attacker of a different species (Thaler et al. 2002). Since plants are commonly attacked by a range of herbivores and pathogens sequentially or simultaneously, molecular mechanisms in response to attack are also selected for under the regime of a community of attackers. Furthermore, plants stand in a community alongside other plants that may provide information on the likelihood of attack. Plants that have initiated an induced response may prime a defence in neighbouring plants (Ton et al. 2007; Frost et al. 2008). These primed plants do not express altered levels of plant defences, but respond more rapidly and/or more intensely than control plants to herbivore attack. The interplay between different molecular mechanisms of defence, therefore, provides important issues in a molecular approach of plant-insect interactions in a community perspective.

tors and parasitoids, they may also extend to effects on vertebrate predators (Bailey et al. 2006; Gruner and Taylor 2006). Furthermore, direct plant defences may affect members of the third trophic level and beyond as mediated through effects on the herbivore (Harvey et al. 2003, 2007). Additionally, direct chemical defences that affect the performance of herbivores, also affect their immune response against parasitoid eggs (Turlings and Benrey 1998; Ferrari and Godfray 2006; Karimzadeh et al. 2008). When herbivore larvae feed on plants with low concentrations of chemical defences, the herbivores perform better and are capable of encapsulating many of the parasitoid eggs. Poorly performing herbivores have reduced egg-encapsulation capacity. Thereby herbivore populations that feed on plants expressing a high level of direct defence may sustain larger parasitoid abundance. On the other hand, adapted herbivores may sequester direct defences of the plant that decrease the performance of parasitoids (Müller et al. 2001). Plant quality, through its effects on herbivores, may further shape parasitoid communities indirectly by a series of trait- and density-mediated interactions among species. For example, two *Brassica oleracea* genotypes that differ in secondary chemistry, leaf thickness and architecture affect the density and body size of aphids differently. This further cascades across trophic levels and affects the diver-

sity of parasitoids (third trophic level) and hyperparasitoids (fourth trophic level), through changes in quantity and quality of the aphids (second trophic level) (Bukovinszky et al. 2008).

Defence in a tritrophic context

Most plants also have mechanisms through which they enhance the effectiveness of carnivores (Schaller 2008; Heil 2008). The presence of carnivores may be promoted by plant structures that offer housing to predators, extra floral nectar that function as food source for predators or herbivory induced plant volatiles (Heil 2008). The latter compose of qualitative or quantitative changes in the mixture of volatiles produced by a plant upon herbivory, which thereby signal reliable information on herbivore availability to parasitoids and predators (Vet and Dicke 1992). This results in indirect defence when plants that produce these compounds attract predators that enhance the predation pressure on their herbivorous attackers (Takabayashi and Dicke 1996; De Moraes 1998; D'Alessandro and Turlings 2006; Heil 2008). More importantly, indirect defences have a genetic basis and plant genotypes are found to differ widely in their production of volatile organic compounds after induction by the same herbivore species (D'Alessandro and Turlings 2006, Heil 2008). Plant genotypes may thereby also directly affect the abundance and diversity of predators and parasitoids. There is ample evidence that plants with increased volatile emissions, either by adding synthetic blends or by genetic modification, attract more parasitoids and predators (Thaler et al. 1999; Kessler et al. 2004; James 2003; James and Price 2004; Kessler and Halitschke 2007). Consequently, these plants represent enemy-dense space to herbivores (James 2003; James and Price 2004). Few studies, however, have indeed shown that genotypes that produce different mixtures of volatiles also differentially attract parasitoids and predators in the field (Bradburne and Mithen 2000; Rassman et al. 2005). Furthermore, plants that emit volatiles upon damage by a particular herbivore may not only attract parasitoids that have a trophic relationship with the herbivore. Plant volatiles may also attract (a) parasitoids and predators that have no trophic relationship with the inducing herbivore, (b) pollinators and (c) other herbivores (De Moraes et al. 2001; Shiojiri et al. 2002; Kaplan et al. 2007; Kessler and Halitschke 2007; Bruinsma and Dicke 2008). Potentially, induced indirect defences may also attract species of the fourth or higher trophic levels, such as hyperparasitoids that feed on larvae of primary parasitoids. It is, however, unknown whether these defensive traits also directly affect the abundance of hyperparasitoids. Even without attraction of hyperparasitoids, indirect defences may affect the species richness and abundance of hyperparasitoids through trophic cascades (Buitenhuis et al. 2005). Indirect defence that promotes larger numbers of primary parasitoids can directly increase the niche availability for hyper-parasitoids and thereby sustain larger hyperparasitoid diversity (Bukovinszky et al. 2008). We conclude that through genetic differences in indirect defences, plant genotypes directly affect the abundance and diversity of predators and parasitoids.

Induced direct defence as a phenotypic effect on community diversity

Induced plant responses are not restricted to indirect extrafloral nectar or volatile-mediated defences, but include changes in plant morphological, allelochemical or nutritional quality. These changes in plant quality may affect the performance of the attacking herbivore as well as that of other species that colonize the plant (Agrawal 2000; Traw and Dawson 2002; Poelman et al. 2008b). Molecular plant biologists and ecologists have established that herbivore species differentially induce responses in plants and that these altered plant phenotypes differentially affect subsequent herbivores (Agrawal 2000; Heidel and Baldwin

2004; de Vos et al. 2005). Since induced responses of plants are generally systemic and may last for an entire season, herbivores that are associated with the same plant may interact with each other through their effect on plant quality. These herbivore interactions include spatially separated herbivores such as those that feed above and below ground (Bezemer and van Dam 2005) or those that are temporally separated. Induced plant responses are now widely accepted to mediate herbivore competition (Faeth 1986; Denno et al. 1995; Kaplan and Denno 2007). The whole suite of induced plant responses not only affects the performance of subsequently colonizing herbivores, but may also affect their host plant selection behaviour (Shiojiri et al. 2002; Long et al. 2007; Poelman et al. 2008b). This implies that induced responses to early-season herbivores may have community-wide effects similar to constitutive defences. Induced plant phenotypes may harbour different communities than control plants. In addition to examples in which interactions between two species have been tested in the field (Martinsen et al. 1998; Denno et al. 2000; Kessler and Balwin 2004), experimental evidence for a role of induced plant responses in shaping entire insect communities is emerging too. For example, experimentally removing an herbivorous beetle from its host plant *Hormathophylla spinosa*, demonstrated that this beetle species strongly affects the abundance of other insect species on the plant (Gonzáles-Megías and Gómez 2003). In brassicaceous plants, experimentally applied initial herbivory by *Pieris rapae* caterpillars affects the abundance of a suite of herbivore species from different guilds (Figure 1) (Agrawal and Sherriffs 2001; Poelman et al. 2008b). Induction of defence responses by applying the hormone JA led to the conclusion that JA-regulated defences play a key role in reducing future attack by a suite of herbivore species (Thaler et al. 2001). It has now been demonstrated in terrestrial and tidal systems that each herbivore species differentially affects the herbivore community (Van Zandt and Agrawal 2004; Viswanathan et al. 2005; Long et al. 2007).

Under natural circumstances, plants are colonized sequentially or simultaneously by a whole suite of attackers. These sequences may lead to a further diversification of induced plant phenotypes that harbour different associated insect communities. Plant responses to initial herbivory are known to interact with responses to subsequent attackers (Thaler et al. 2002; Paul et al. 2000; Viswanathan et al. 2007; Zarate et al. 2007). The response to initial herbivory may enhance the defence response upon future attack (de Vos et al. 2006; Poelman et al. 2008b). Conversely, negative cross talk between signal-transduction pathways may inhibit the response to a second herbivore (Thaler et al. 2002, Viswanathan et al. 2007; Rasmann and Turlings 2007), although it was also found that plants are able to redirect the defence response towards the second herbivore (Voelckel and Baldwin 2004; Poelman et al. 2008b). It remains to be investigated whether particular types of herbivores elicit responses in plants that lead to canalization of plant responses or that overrule responses to initial attack by other species. Thereby some herbivore species may modify plant phenotypes with more profound consequences for the associated insect community than other herbivores.

Responses of generalists and specialists: consequences for community composition

Herbivores have been reported to compete asymmetrically with each other through induced plant responses (Inbar et al. 1999; Agrawal 2000; Stotz et al. 2000; Long et al. 2007). Some species induce plant responses that affect many other species, whereas other herbivores elicit responses that only affect their own performance or that of a limited number of other species. Herbivores inducing responses affecting a wide range of subsequent colonizers strongly drive the composition of a community. They can be seen as key-stone herbivores (Hunter 1992). Herbivores may have a different effect on the future composition of

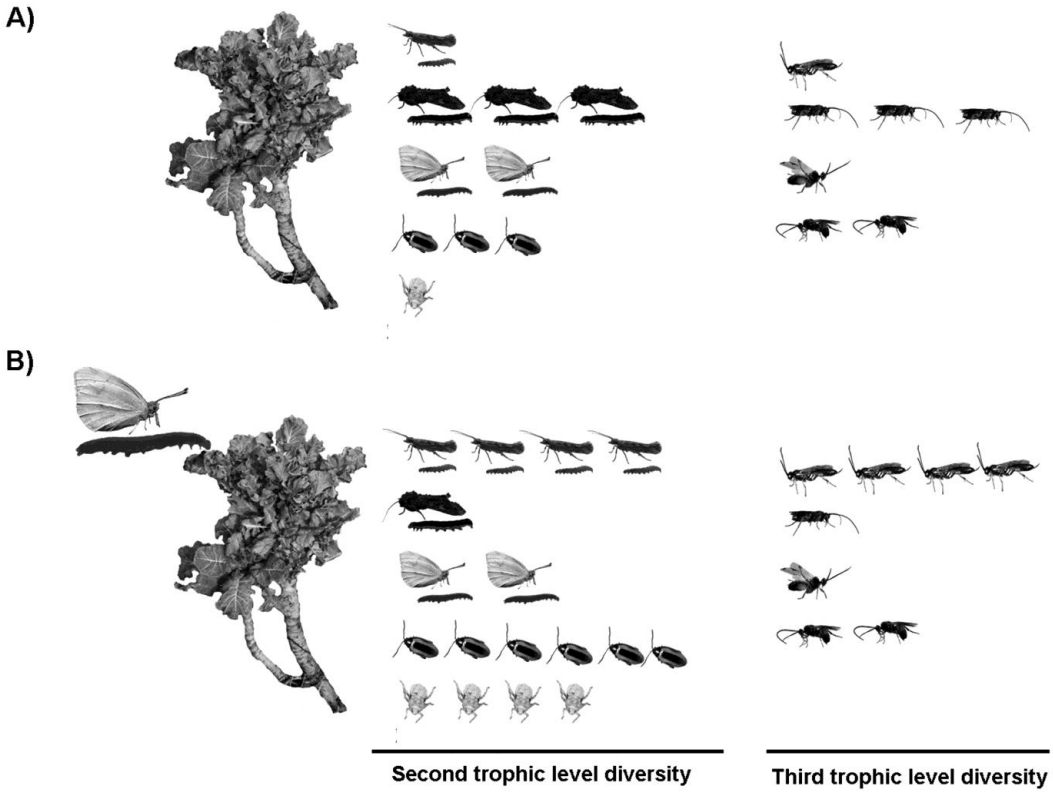

Figure 1. Early-season herbivory by the specialist *Pieris rapae* induces plant responses in *Brassica oleracea* that promote the attraction of specialist herbivores and repel generalist herbivores. Control plants (A) with constitutive defences harbour a community with a higher relative abundance of generalist herbivores, whereas the same plants that are induced by *P. rapae* herbivory (B) harbour an herbivore community that is dominated by specialists. The effect of induced defence has a cascading effect on diversity on the third throphic level. The number of individuals of the species depicted reflects their abundance. Early season herbivores induce plant responses that affect the preference and performance of subsequent colonizers differentially. Thereby an early season herbivore affects the abundance and diversity of the entire insect community. Specialist herbivores are increasingly found to be strong plant inducers and to strongly prefer induced plants.

the community by their difference in the mode of feeding that elicits a different response in the plant (De Vos et al. 2005; Mewis et al. 2006). Across feeding guilds, however, herbivores differ in host plant range and those with a narrow range restricted to a single plant family are termed specialists. These species are adapted to host plant family specific characteristics such as defence chemicals that are typically harmful to generalists (Jaenike 1990; Schoonhoven et al. 2005). When experimentally inducing plants with either specialist or generalist herbivores, regardless of feeding guild specialist herbivores have been identified as strong drivers of insect communities through induced plant responses, whereas generalists seem not to induce such effects (van Zandt and Agrawal 2004; Viswanathan et al. 2005).

Although specialist herbivores may be strong drivers, an important question is: which species are responding to the changes in plant phenotypes? Both in research on constitutive and inducible defences the debate currently centers around the question whether some groups of herbivores show similarities in the nature of their reactions. Although her-

bivore species often covary in response to variation in plant traits (Leimu and Koricheva 2006; Johnson and Agrawal 2007), host-plant-specific chemicals affect generalist and specialist attackers differently and thereby affect insect community composition (Whitham et al. 2006; Kaplan and Denno 2007). In several studies it has been found that induced plant responses, after early-season herbivory, differentially affected host plant acceptance by subsequently arriving herbivores that differ in host plant range (Martinsen et al. 1998; Shiojiri et al. 2002; Kessler et al. 2004; Rodriguez-Saona et al. 2005; Viswanathan et al. 2005; Johnson and Agrawal 2005, 2007; Long et al. 2007; Inbar and Gerling 2008). In the seaweed *Fucus vesiculosus*, specialist herbivory suppressed populations of subsequently colonizing generalists, which was caused by a preference of generalists for undamaged seaweed (Long et al. 2007). Other studies documented that generalist herbivores avoid induced plants, but more importantly, observed specialists to be more abundant on induced plants (Martinsen et al. 1998; Agrawal and Sherriffs 2001; Shiojiri et al. 2002; Poelman et al. 2008b). There is increasing evidence that herbivores with a similar host-plant range covary in their reactions to induced plant responses, i.e. across feeding guilds specialist herbivores prefer induced plants. Induction of plant responses by a specialist herbivore drives the herbivore community more profoundly than plant induction by a generalist and results in a community balanced towards specialists (Figure 1).

Interplay between constitutive and induced direct and indirect defences

The identification of strong drivers of community composition that result in a covariation in herbivore responses shows that herbivores do not act as independent agents of selection on plant defences (Maddox and Root 1990; Dungey et al. 2000). Plant responses to a particular herbivore species have community-wide effects and determine the likelihood of future attack by a particular group of attackers. The emerging pattern of specialist herbivores acting as strong drivers of the future composition of an herbivore community biased to a specialized community has strong implications for induced defences. When a community of attackers is balanced towards generalist attackers that are less likely to drive community composition by their effect on plant quality, plants may be selected to have high levels of constitutive defences that repel or deter generalists. When the probability of any attack is relatively small or damage can be compensated by regrowth, plants surrounded by a community of generalist attackers may be favoured by induced defences. Plants gain a fitness benefit by saving costs of defence when herbivory is absent and only produce defences that tackle the current attacker and reduce future attack by generalists (Heil and Baldwin 2002). However, when plants grow in environments where specialists are more abundant, induced defences may be non-adaptive. Attack by specialists strongly induces these defences and consequently other specialists are likely to colonize these plants. When the induced responses of the plant result in effective attraction of predators of the specialist herbivores, induced defences may be beneficial (Kahl et al. 2000).

However, even when particular groups of herbivores covary in their response to constitutive or induced defences, each of the herbivores may have a different impact on the plant. Some herbivores are more destructive to plants than other herbivores. For example, large caterpillars may consume an entire plant or prefer to feed on reproductive organs (Smallegange et al. 2007) and, consequently, impose stronger selection pressure than herbivores that only consume a few leaves. When species that impose relatively little damage to plants drive the community composition to the extent that more destructive species are less likely to colonize the plant (Kessler and Baldwin 2004), plants may be favoured to accommodate these species as first attacker. When plants actively promote the presence of these species, for example by synchronisation of their phenology, they may ‘vaccinate’

themselves against the more ravaging attackers (Kessler and Baldwin 2004). Thus, plant defence strategies are strongly dependent on the phenology and composition of the local insect community, because the community responses to certain phenotypes act as selective agent of plant defences. The tight link between plant phenotype and insect community as a whole should, therefore, become a central issue in addressing community ecological and molecular aspects of plant sciences (Kessler and Halitschke 2007; Bruinsma and Dicke 2008).

Future perspectives

The interplay between genetic variation and phenotypic plasticity in direct and indirect plant defences and their effects on higher trophic level biodiversity links exciting research fields. Although many of the studies reviewed here provide evidence of plant-mediated effects on community composition, all studies have primarily focused on herbivores and community-wide cascading effects through herbivores. A critical gap in our knowledge of the effect of plant genotype in structuring insect communities is whether indirect defences lead to community-wide effects that are independent of a trophic cascade via effects on herbivores. When indirect defences do play such a role in structuring communities, their relative contribution compared to direct defences should be assessed to evaluate the benefit of each defence strategy to the plant. Although plant genotypes are known to harbour distinct insect communities, early-season herbivores, through induced plant responses, can be drivers of community diversity too. These recent advances in our understanding of plant-mediated herbivore interactions underpin the need to investigate whether plant genotype is more important for insect community composition than the induction by early-season herbivores. To assess the relative importance of genotype and induced phenotype in the field, it is therefore important to identify whether herbivore induction converges a range of genotypes into more similar phenotypes or whether induction leads to further divergence. In addition, it must be established whether all plant genotypes are likely to be colonized by the same early-season herbivores and whether sequential herbivory eventually leads to convergence of phenotypes. The apparent consensus that specialist herbivores are strong drivers of communities has only recently emerged and needs to be rigorously tested in a wider range of natural plant systems. Information is needed to identify to what extent herbivores differ in their response to particular constitutive and induced defences and whether there are herbivores that have a relatively strong influence on the community (key-stone species). When studying particular defence traits in a certain plant species it is essential to understand the phenology of the community of attackers before testing the effect of a trait on a particular herbivore species. The ultimate challenge is to eventually link selection on specific plant defence traits to community-wide effects and to establish how this community as a whole imposes selection on plant traits. Addressing these exciting issues will greatly benefit from a multidisciplinary approach that includes studies at different levels of biological organisation, from gene expression to phenotype expression and community dynamics.

Outline of this thesis

The current thesis is the result of a PhD project that was part of a programme that addressed the role of plant defence in shaping biodiversity of insect communities. The project aimed at identifying the plant traits that affect higher trophic levels by integrating a transcriptomics, metabolomics and ecological approach. Cultivated varieties of white cabbage (*Brassica oleracea* var *alba*) and their associated insect community were used as a model system, which allowed a transcriptomic approach by using the available micro-arrays

based on the genome of the model plant *Arabidopsis thaliana*. This well-studied plant is a member of the Brassicaceae and its genome is 85% homologous to *Brassica oleracea* (Lee et al. 2004). Furthermore, the interaction between *B. oleracea* and its herbivorous insects has been studied extensively (Root 1973; Moyes et al. 2000; Gols et al. 2008b). Cultivars have been selected for homogeneity and, therefore, show little variation for plant traits within a cultivar, in contrast to the large variation found within wild populations of *B. oleracea* (Gols et al. 2008b). Between cultivars, however, clear differences in phytochemistry, including herbivory induced volatile emission have been reported (Olsson and Jonasson 1994; Geervliet et al. 1997). Cultivars can, therefore, be used as a proxy for genotypic variation in these resistance traits and can, thus, be studied for differential effects on insect communities.

To understand the complexity of interactions between and among organisms of several trophic levels it is essential to study the reciprocal effects of organisms on each other. Here, I use a multidisciplinary approach by assessing transcript and metabolic changes in the plants caused by herbivorous insects. The effect of plant characteristics on herbivorous insects as well as higher trophic levels is studied under controlled laboratory conditions and in the field. This is done by focusing on interactions between two species as well as by assessing effects on community composition. Multidisciplinary approaches have proven to advance the field of plant insect interactions significantly (Kessler et al. 2004; Bruinsma and Dicke 2008; Zheng and Dicke 2008).

In Chapter 2, I present laboratory experiments on the variation in direct plant resistance of *Brassica oleracea* cultivars against herbivorous attackers. The foliar glucosinolate composition for eight cultivars was analyzed and the resistance level of the cultivars was assessed by measuring the performance of specialist and generalist herbivores. In particular, the hypothesis is tested that high levels of glucosinolates affect the performance of a generalist herbivore (*Mamestra brassicae*) and that these compounds are less effective against specialists (*Pieris rapae, Plutella xylostella*; Ratzka et al. 2001; Wittstock et al. 2004). In addition, I assessed the responses of plants elicited by the three herbivore species to study whether plants alter their foliar glucosinolate concentration differentially after attack by different herbivore species. Furthermore, this chapter addresses the correlation between induced changes in glucosinolate concentration and herbivore performance.

Based on the results described in Chapter 2, I selected two cultivars that are resistant against herbivores and two cultivars that are more susceptible to herbivores from the total set of eight cultivars. In Chapter 3, the four cultivars were used to study the role of direct plant resistance in structuring the herbivorous insect community. In the field it is tested whether the large qualitative variation in glucosinolates of the cultivars correlates with the composition of the herbivore community. Here, I focused on the degree of similarity in reaction of specialist and generalist herbivores to cultivars and studied host plant choice by a specialist (*Pieris rapae*) and a generalist (*Mamestra brassicae*) herbivore under laboratory conditions.

Chapter 4 addresses the volatile-mediated indirect resistance of the cultivars. Under controlled laboratory conditions, I collected headspace samples of the cultivars and compared the emission of herbivore-induced plant volatiles. In two-choice tests the attractiveness of the cultivars to parasitoid wasps (*Cotesia glomerata* and *C. rubecula*) was tested when the plants were under attack by a host (*P. rapae*) of the parasitoids. Furthermore, the relative attraction of parasitoids by the cultivars was also investigated under field conditions. In Chapter 5 the ranking of attractiveness of cultivars to primary parasitoids found in Chapter 4 is used to study the role of volatiles in structuring parasitoid communities. The experiments tested whether cultivars with high indirect resistance also attract more

hyperparasitoids that parasitize cocoons of *Cotesia* wasps. Hyperparasitoid communities of the gregarious parasitoid *Cotesia glomerata* and the solitary parasitoid *C. rubecula* on the different cultivars are compared. In this chapter I address whether solitary and gregarious primary parasitoids differ in vulnerability to hyperparasitoid attack and whether herbivory-induced volatiles play a role in the hyperparasitoid pressure on *Cotesia* wasps.

Having identified the effects of direct and indirect plant resistance on the structure of insect communities, I studied whether induction by a particular insect species affects the plant-associated insect community. Chapter 6 addresses whether induced plant responses to herbivory by the specialist *P. rapae* affect future plant responses to subsequent attackers and, consequently, influence the behaviour of herbivorous attackers. The expression of four direct-defence-related genes in response to herbivory by *P. rapae* and subsequent secondary attack by a generalist (*M. brassicae*) or a specialist herbivore (*Plutella xylostella* or *P. rapae*) is studied. The effect of *P. rapae*-induced plant responses is further studied for the performance and host plant acceptance of the three herbivore species in the laboratory as well as in the field. In Chapter 7, I studied whether the plant responses to herbivory by *P. rapae* early in the season result in herbivore community-wide effects later in the season. This chapter addresses whether *P. rapae*-induced plants and uninfested control plants harbour different herbivore communities and I studied whether particular groups of herbivores covary in their response to induced plant defences.

Finally, in Chapter 8 the findings of this thesis are discussed, with an emphasis on an integration with results from transcriptomic studies executed in the other PhD-project within the research programme. Here I focus on how variation in plant defensive characteristics affects the plant-associated insect community and how the community in its turn affects the plant phenotype. It is emphasized that plant traits and insect communities affect each other reciprocally and that these interactions underlie plant-associated biodiversity. Finally, I present an outline for future directions in disentangling plant-mediated effects on insect diversity by using multidisciplinary approaches.

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Chapter 2

Picture: Ciska Raaijmakers

Performance of specialist and generalist herbivores feeding on cabbage cultivars is not explained by glucosinolate profiles

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Abstract

Plants display a wide range of chemical defences that may differ in effectiveness against generalist and specialist insect herbivores. Host plant-specific secondary chemicals such as glucosinolates in Brassicaceae typically reduce the performance of generalist herbivores, whereas specialists have adaptations to detoxify these compounds. The concentration of glucosinolates may also alter upon herbivory, allowing the plant to tailor its response to specifically affect the performance of the attacking herbivore. We studied the performance of three Lepidoptera species, two specialists [*Pieris rapae* L. (Pieridae), *Plutella xylostella* L. (Yponomeutidae)] and one generalist [*Mamestra brassicae* L. (Noctuidae), when feeding on eight cultivars of *Brassica oleracea* L. and a native congener (*Brassica nigra* L.) and related this to the glucosinolate (GS) content. We tested the hypotheses (1) that a generalist herbivore is more affected by high glucosinolate concentrations and (2) that generalist feeding has a stronger effect on glucosinolate levels. Although performance of the three herbivores was different on the *B. oleracea* cultivars, *M. brassicae* and *P. xylostella* had a similar ranking order of performance on the eight cultivars. In most of the cultivars, the concentration of indole GS was significantly higher after feeding by *P. rapae* or *M. brassicae* than after *P. xylostella* feeding. As a consequence, the total concentration of GS in the cultivars showed a different ranking order for each herbivore species. The generalist *M. brassicae* performed equally well as the specialist *P. xylostella* on cultivars with high concentrations of GS. Our findings suggest that secondary metabolites other than glucosinolates or differences in nutrient levels affect performance of the species studied.

Key words: induced response, plant defence, Lepidoptera, Pieridae, Yponomeutidae, Noctuidae, *Pieris rapae, Plutella xylostella, Mamestra brassicae*

Introduction

Within the plant kingdom, a wide range of morphological and chemical defences against insect herbivory has evolved (Zangerl and Berenbaum 1990; Cipollini et al. 2003). As a result of frequency-dependent selection by a community of attackers that is highly variable in time and space and shows variable susceptibility to plant defences, the variation in defence expression is often maintained within a single plant species (Mithen et al. 1995; Nielsen 1997; van Leur et al. 2006). Each attacker may select for a specific set of defence traits in the plant and each expressed defence trait may have different effects on herbivores that differ in their susceptibility to particular defence chemicals (Jaenike 1990; van der Meijden 1996).

In brassicaceous plants, the characteristic secondary chemicals, glucosinolates (GS) (Fahey et al. 2001), and their breakdown products are well known to effectively decrease performance of generalist herbivores (Chew 1988; Olsson and Jonassen 1994; Traw and Dawson 2002; Agrawal and Kurashige 2003). The same chemicals can also reduce performance of specialist herbivores (Agrawal 2000; van Dam et al. 2000), even though such chemicals attract these specialists or stimulate them to feed (David and Gardiner 1966; van Loon et al. 1992, 2002; Renwick 2002). These specialists, however, may be able to neutralize the defences present in their host plants. For example, toxic compounds may be rapidly excreted, hydrolyzed, or enzymatically disarmed (Ratzka et al. 2002; Wittstock et al. 2004). Some specialist herbivores even sequester plant defence compounds for their own defence (Schoonhoven et al. 2005; Després et al. 2007) and may be highly tolerant to these compounds (Müller and Sieling 2006).

Moreover, the concentration of foliar GS has been found to change after herbivory in various *Brassica* species (Griffith et al. 1994; Siemens and Mitchell-Olds 1998; van Dam et al. 2004). These induced responses of plants (Karban and Myers 1989) may have evolved as an adaptive trait to reduce the costs of defence when herbivores are absent, and may also enable plants to respond specifically to the type of attacker (Karban and Baldwin 1997; Zangerl 2003). Plants may recognize the herbivore that is feeding, based on the pattern of imposed damage and the salivary constituents, creating an opportunity for a fine-tuned response by the plant (Mattiacci et al. 1995; Dicke 1999; Kahl et al. 2000). Consequently, it may be expected that specialists and generalists have different effects on plant responses. Differential responses by plants to different herbivore species have been found in Brassicaceae (Agrawal 2000; Traw and Dawson 2002; Mewis et al. 2006). However, induced plant responses did not always result in induced resistance against the inducing herbivore itself and generalists were not more susceptible than specialists to induced responses of the plant (Agrawal 2000). Furthermore, plants have also been found to respond similarly to generalist and specialist herbivores (Reymond et al. 2004). There is limited information on the nature of differential responses of plants to herbivory that also result in enhanced resistance against the attacker.

Here we studied the relationship between specificity in defence pattern expression and the level of specialization of the attacking herbivore. We tested the effect of GS composition and concentration of brassicaceous plants on the performance of three Lepidoptera species and tested whether the host plant specialization level of the herbivores correlates with GS concentrations before and after herbivore attack. *Pieris rapae* L. (Pieridae) and *Plutella xylostella* L. (Yponomeutidae) were studied to represent specialist species; both have overlapping host plant ranges within the Brassicaceae. *Mamestra brassicae* L. (Noctuidae) was used as a generalist, having a host-plant range extending beyond the Brassicaceae. We quantified constitutive concentrations of GS in eight different cultivars of *Brassica oleracea* L. and a native congener (*Brassica nigra* L.) and measured GS concen-

trations after herbivory by caterpillars of the three species. Both within and between wild species as well as crops, there is a vast diversity of GS structures and concentrations (Benrey et al. 1998; Kliebenstein et al. 2002), which differ in their effectiveness as defence compounds (Fahey et al. 2001). The eight cultivars were all selected from the *alba* group to reduce morphological variation among cultivars and we selected a mix of open pollinated cultivars and more recently cultivated F1 hybrids from different plant breeders to enhance variation in chemical composition between cultivars. We used *B. nigra* as a reference species, representing a species that has a high level of direct defence with total GS concentrations 3-5-fold higher than found in cultivated plants (Mithen et al. 1995).

We specifically tested the hypothesis that performance of the generalist will be better on the *Brassica* species and cultivars containing low GS concentrations while we expected no effect on the specialists, predicting that the two specialists are more similar to each other than to the generalist in their rank order of performance. We investigated 1) whether performance of the three herbivore species correlates with the amount of glucosinolates and 2) whether a particular herbivore affects a particular set of GS consistently in different cultivars of a single plant species. The implications of our results for the concept of specificity of plant-herbivore interactions are evaluated.

Materials and methods

Plants and insects

The following eight cultivars of white cabbage (*B. oleracea* var. *alba*) were used (sources are given between brackets): Domia (Horticulture Research International, Warwick, UK); Badger Shipper, Jersey Queen, and Christmas Drumhead (Centre for Genetic Resources, CGN, Wageningen, The Netherlands), representing open pollinated cultivars, and Lennox, Rivera, and Bartolo (Bejo Zaden BV, Warmenhuizen, The Netherlands) and Stonehead (Sakata Holland BV, Rijsenhout, The Netherlands), representing more recently cultivated F1 hybrids. Seeds of *B. nigra* were collected in 2000 from a population near Heteren, The Netherlands (51°57`N, 5°45`E) and propagated by open pollination several times since. All plants were grown in a climatized glasshouse compartment. Seeds were germinated (15 November 2004) on peat soil (Lentse potgrond, No. 4, Lent, The Netherlands) and 10 days later individual seedlings were transferred to peat soil in 1.45 l pots. Plants were provided with SON-T light (500 µmol/m^2/s; L16:D8), 18–26 ºC, and 50-70% r.h. When the plants were 4 weeks old, they were fertilized by applying Kristalon Blauw (Hydro Agri Rotterdam, The Netherlands) (N-P-K) 19-6-20-3 micro (2.5 mg/l) to the soil and this was repeated once every other week during the experiment. Pots with 8-week-old plants were placed individually in trays containing a water layer of 1 cm at the start of the experiment and this water level was maintained during the experiment (see below).

Herbivore performance

On 10 January 2005 (t = 0), the newest fully grown leaf of 15 plants of each cultivar was sampled for glucosinolate (GS) analysis, stored on ice and transferred to a -80 ºC freezer. Just after the leaves had been collected, the plants were randomly assigned to one of three infestation treatments: *P. rapae*, *P. xylostella*, or *M. brassicae*. All caterpillars were obtained from the respective insect cultures of the Laboratory of Entomology, Wageningen University and were cultured on *B. oleracea* var. *gemmifera* cv. Cyrus. Each plant was infested with 10 neonate caterpillars of the herbivore species and with five replicates per cultivar-herbivore species combination. Distance between plants and the 1-cm layer of water in the trays prevented caterpillars from moving between plants. After 6 days (t = 1), a second newest fully grown leaf, on which caterpillars had been feeding, was collected

following the same procedure as mentioned at the beginning of this section. The herbivores were collected, weighed individually to the nearest 0.1 mg, and placed back onto the plants from which they originated. On day 12 (t = 2), we collected a third fully grown leaf that was damaged by the caterpillars and was positioned as first leaf below the leaf collected on day 6. The caterpillars were surveyed daily for survival and development until pupation. When caterpillars had pupated, the date of pupation was noted and pupae were weighed to the nearest 0.1 mg. Caterpillars of *M. brassicae* pupate in the soil. Therefore, caterpillars of *M. brassicae* that reached the final instar were placed in plastic boxes containing a layer of 5 cm of peat soil and were provided ad libitum with excised leaf material of the plant they originated from until pupation.

The performance experiment was repeated starting on 6 April, 2005 with a new set of 8-week-old plants. Ten first instars were placed on each of 10 plants for each cultivar (using 100 caterpillars per cultivar), i.e., twice as many as in the first experiment, and surveyed for growth and development. As there were 900 caterpillars per species, on 1 day we could only perform weighings of a single caterpillar species. Therefore, caterpillars were weighed when the fastest growing caterpillar of that particular species reached the fourth instar. This resulted in weighing of *P. xylostella* on day 7, *P. rapae* on day 9, and *M. brassicae* on day 20 since the larvae had been introduced on the plants. For these caterpillars, we also measured the pupal weight and number of days to reach pupation since introduction on the plants.

Glucosinolate profiling

The collected leaves that originated from 135 plants, consisting of eight cultivars and *B. nigra* that were each sampled at three time intervals, were freeze-dried and ground to a fine powder. Fifty mg of ground leaf material per sample was dissolved in methanol. The extract was desulphatased on a DEAE-Sephadex A25 column and the GS content was assessed by high performance liquid chromatography (HPLC), using the method described by van Dam et al. (2004). Five concentrations of sinigrin (sinigrin monohydrate; Sigma, St. Louis, MO, USA) were desulphatased following the same protocol as the samples and were used as an external standard. Glucosinolate detection was performed with a PDA detector (200–350 nm) with 229 nm as the integration wavelength. We used the correction factors at 229 nm from Buchner (1987) and the EC (EC, 1990) to calculate the concentrations of the glucosinolates. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with standards kindly provided by M. Reichelt, Max Planck Institute for Chemical Ecology, Jena (Germany) and a certified rapeseed standard (code BCR-367R; Community Bureau of Reference, Brussels).

Statistical analysis

Performance parameters of each herbivore species were analyzed using SPSS 12.0.1 (Chicago, IL, USA) by running separate tests for each parameter per herbivore species. ANOVA with post-hoc Tukey tests for cultivar comparisons were applied to analyze caterpillar and pupal mass. Kruskal-Wallis tests were applied to analyze the time until caterpillars pupated on different cultivars. Data of the two replicates of the performance experiment were analyzed separately, because caterpillars from the second series were growing slightly faster and were weighed at different time points compared to the first series. Nevertheless, ranking of cultivars for performance of each caterpillar species was similar for both series. Because a larger number of caterpillars were weighed in the second series, we only present detailed herbivore data of this experiment.

The quantitative and qualitative GS content of the plants and the effect of each

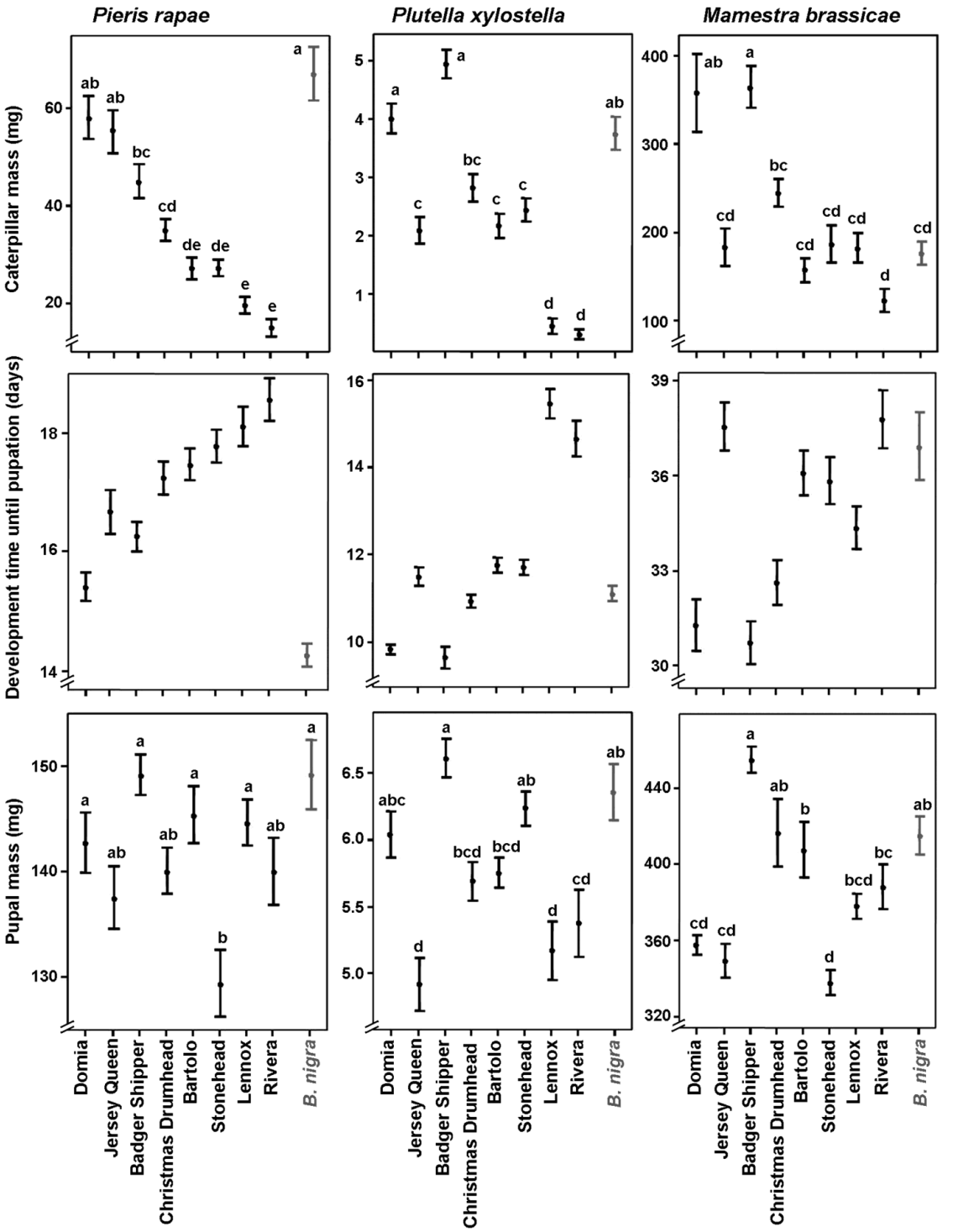

Figure 1. Performance of three herbivore species (*Pieris rapae*, *Plutella xylostella*, and *Mamestra brassicae*) on different cultivars of *Brassica oleracea* (black) compared to the wild species *Brassica nigra* (grey). Per herbivore species from top to bottom of the graph the panels present mean body mass (± SEM; n = 9-76) of caterpillars, time until pupation, and pupal mass. The cultivar sequence was arbitrarily based on caterpillar mass of *Pieris rapae*.

herbivore species on GS content were analyzed using two models. In both models, the dependent variable, GS concentration, was normalized by an arc sin square root transformation. To analyze the constitutive differences in GS profile of the eight *B. oleracea* cultivars and *B. nigra*, we applied a MANOVA on the amount of GS without any herbivore damage (t = 0). To analyze the effect that different herbivore species had on the amount of GS in different *Brassica*-species, a mixed model with repeated measurement structure subjected to plants (6 and 12 days) was used. The GS concentration was modelled by the factors cultivar, herbivore species, time, and their interactions by using the PROC MIXED function of SAS 9.1. Average total-, alkenyl-, and indolyl-glucosinolate concentrations of the cultivars were correlated with average caterpillar mass per cultivar for each herbivore species, using linear regression.

Results

Herbivore performance

We found that caterpillar performance of the three herbivore species depended on the *Brassica* cultivar on which they were feeding, which is reflected in the caterpillar mass (ANOVA, *P. rapae*: $F_{8,550} = 28.039$, $P<0.001$; *P. xylostella*: $F_{8,255} = 31.728$, $P<0.001$; and *M. brassicae*: $F_{8,278} = 9.502$, $P<0.001$). The development time until pupation of these caterpillars showed a reversed pattern; caterpillars that fed on a cultivar resulting in low caterpillar mass had a longer development time (Kruskal-Wallis test, *P. rapae*: 113.644, d.f. = 8, $P<0.001$; *P. xylostella*: 135.759, d.f. = 8, $P<0.001$; and *M. brassicae* 68.217, d.f. = 8, $P<0.001$) (Figure 1). Although the ranking order of performance over the *Brassica*-cultivars and species tested was relatively similar for all three herbivores, post-hoc Tukey tests for cultivar effects on fourth instar mass also revealed differences between the herbivores. The two *Brassica* specialists (*P. xylostella* and *P. rapae*) performed well on *B. nigra*, which had the highest concentration of GS, whereas the generalist *M. brassicae* had a low performance on *B. nigra*. Furthermore, the cultivar Jersey Queen supported lower performance of *M. brassicae* and *P. xylostella* than of *P. rapae*. Over the eight *B. oleracea* cultivars tested, the specialist *P. xylostella* and generalist *M. brassicae* were similar in the ranking order of performance and differed from the specialist *P. rapae* (Figure 1).

Pupal masses of all three herbivore species correlated negatively with the number of days before pupation occurred (Pearson correlation coefficient, *P. rapae*: $r = -0.252$, $P<0.001$; *M. brassicae*: $r = -0.194$, $P = 0.007$; and *P. xylostella*: $r = -0.373$, $P<0.001$). The cultivar on which the caterpillars were feeding affected pupal mass significantly in all three herbivores (ANOVA, *P. rapae*: $F_{8,352} = 5.682$, $P<0.001$; *P. xylostella*: $F_{8,187} = 9.392$, $P<0.001$; and *M. brassicae*: $F_{8,182} = 18.187$, $P<0.001$). Post-hoc Tukey tests revealed that feeding on cultivar Domia resulted in significantly lower pupal masses for *M. brassicae* whereas for *P. rapae* and *P. xylostella*, pupal mass on this cultivar was high or intermediate, respectively. On cultivar Stonehead, *M. brassicae* and *P. rapae* had low pupal masses, but *P. xylostella* reached a relatively high pupal mass (Figure 1).

Plant glucosinolate levels before and after herbivory

The eight *B. oleracea* cultivars and *B. nigra* differed in their constitutive composition of foliar glucosinolates. Levels of glucosinolates of 8-week-old plants (t = 0) were both qualitatively and quantitatively different between cultivars (MANOVA Pillai`s trace: $F_{8,124} = 8.03$, $P<0.001$; glucoiberin (IBE): $F_{8,124} = 9.98$; sinigrin (SIN): $F_{8,124} = 93.00$; progoitrin (PRO): $F_{8,124} = 9.05$; glucoraphanin (RAPH): $F_{8,124} = 6.16$; gluconapin (GNA): $F_{8,124} = 10.88$; 4-hydroxyglucobrassicin (4OHGBC): $F_{8,124} = 13.14$; glucobrassicin (GBC): $F_{8,124} = 20.97$; 4-methoxyglucobrassicin (MGBC): $F_{8,124} = 9.36$; neo-glucobrassicin (NEOGBC):

Table 1. Foliar glucosinolate profiles of 8-week-old plants of eight white cabbage cultivars of *Brassica oleracea* and the wild species *Brassica nigra*. Concentrations (µmol g^{-1} dry weight) of individual glucosinolates (SE in parentheses) are averages of 15 plants and differ significantly between cultivars (MANOVA Pillai`s trace: $F_{8,124} = 8.026$, $P<0.001$ for all compounds). Scientific compound names are given for side chains only and all are complemented with –glucosinolate in their full scientific name

		Brassica oleracea var. *alba*				
Compound	Common name	Domia	Jersey Queen	Badger Shipper	Christmas Drumhead	
Alkenyl glucosinolates						
3-methylsulfinylpropyl	Glucoiberin (IBE)	1.41 (0.40)	1.45 (0.62)	1.67 (0.47)	0.12 (0.07)	
2-propenyl	Sinigrin (SIN)	1.57 (0.33)	1.33 (0.36)	0.58 (0.15)	0.33 (0.09)	
R-2-hydroxy-3-butenyl	Progoitrin (PRO)	0.01 (0.01)	0.56 (0.21)	0.46 (0.19)	-	
4-methylsulfinylbutyl	Glucoraphanin (RAPH)	0.04 (0.03)	3.17 (1.58)	1.10 (0.35)	0.01 (0.00)	
3-butenyl	Gluconapin (GNA)	0.13 (0.03)	0.82 (0.25)	0.19 (0.07)	0.06 (0.03)	
Indolyl glucosinolates						
4-hydroxy-3-indolylmethyl	4-hydroxyglucobrassicin (4OHGBC)	0.05 (0.01)	0.23 (0.05)	0.13 (0.02)	0.04 (0.01)	
3-indolylmethyl	Glucobrassicin (GBC)	0.09 (0.02)	1.22 (0.30)	0.24 (0.04)	0.08 (0.02)	
1-methoxy-3-indolylmethyl	Neo-glucobrassicin (NEOGBC)	0.01 (0.00)	0.03 (0.01)	0.33 (0.14)	0.02 (0.01)	
4-methoxy-3-indolylmethyl	4-methoxyglucobrassin (MGBC)	0.08 (0.01)	0.10 (0.03)	0.09 (0.01)	0.02 (0.01)	
Aromatic glucosinolates						
2-phenylethyl	Gluconasturtiin (NAS)	-	-	-	-	
Total GS		3.38 (0.74)	8.91 (2.74)	4.79 (0.88)	0.67 (0.19)	

						Brassica nigra
Compound	Common name	Bartolo	Stonehead	Lennox	Rivera	
Alkenyl glucosinolates						
3-methylsulfinylpropyl	Glucoiberin (IBE)	1.20 (0.48)	0.01 (0.01)	0.83 (0.26)	0.03 (0.02)	-
2-propenyl	Sinigrin (SIN)	0.68 (0.14)	3.72 (0.57)	0.42 (0.06)	0.15 (0.05)	28.33 (3.42)
R-2-hydroxy-3-butenyl	Progoitrin (PRO)	0.03 (0.02)	0.01 (0.01)	-	0.04 (0.02)	-
4-methylsulfinylbutyl	Glucoraphanin (RAPH)	0.14 (0.08)	-	0.08 (0.04)	0.01 (0.01)	-
3-butenyl	Gluconapin (GNA)	0.05 (0.02)	0.07 (0.02)	-	0.06 (0.02)	-
Indolyl glucosinolates						
4-hydroxy-3-indolylmethyl	4-hydroxyglucobrassicin (4OHGBC)	0.09 (0.02)	0.01 (0.00)	0.11 (0.02)	0.09 (0.03)	0.26 (0.05)
3-indolylmethyl	Glucobrassicin (GBC)	0.20 (0.04)	0.03 (0.01)	0.11 (0.01)	0.16 (0.03)	0.13 (0.05)
1-methoxy-3-indolylmethyl	Neo-glucobrassicin (NEOGBC)	0.01 (0.01)	-	0.02 (0.01)	0.01 (0.00)	-
4-methoxy-3-indolylmethyl	4-methoxyglucobrassin (MGBC)	0.02 (0.00)	0.02 (0.00)	0.02 (0.01)	0.10 (0.04)	0.09 (0.03)
Aromatic glucosinolates						
2-phenylethyl	Gluconasturtiin (NAS)	-	-	-	-	0.14 (0.04)
Total GS		2.42 (0.66)	3.85 (0.61)	1.58 (0.34)	0.64 (0.16)	28.95 (3.47)

$F_{8,124}$ = 15.80; and gluconasturtiin (NAS): $F_{8,124}$ = 24.83, all P<0.001), with *B. nigra* having a 2-3-fold higher amount of total GS than the *B. oleracea* cultivars (Table 1). After 6 and 12 days of herbivore feeding, the GS levels of the cultivars were still significantly different for each compound (Table 2) and the concentration of all compounds had increased 2-5-fold. Furthermore, feeding by the three different caterpillar species resulted in different levels of all indole GS compounds measured (Table 2, Figure 2). For two of the indole compounds (4OHGBC and GBC), there was an interaction between caterpillar species and the cultivar on which they had been feeding. For most of the cultivars, feeding by *P. rapae* and *M. brassicae* resulted in higher amounts of indole GS, except for cultivar Christmas Drumhead. The amount of four compounds (PRO, GNA, 4OHGBC, and MGBC) increased only marginally between 6 and 12 days of herbivore feeding (Table 2). For alkenyl GS compounds, no differences were found between caterpillar species with the exception of an interaction between cultivar and caterpillar for sinigrin (Table 2). None of the performance parameters correlated with glucosinolate concentration of the *Brassica*-species and all three herbivores performed poorest on cultivar Rivera, which has low concentrations of glucosinolates when grown under greenhouse conditions (Table 3).

Discussion

Our study revealed that the difference between caterpillar performance on native and cultivated plants as well as the ranking order of performance for *B. oleracea* cultivars varied for the three herbivore species. An increase in the number of days until pupation generally coincided with lower pupal mass. Performance differences were already reflected in caterpillar mass differences when the caterpillars of a particular species reached the fourth instar. However, in contrast to our hypothesis that plant defence affects generalists and specialists differently, the generalist *M. brassicae* and the specialist *P. xylostella* were similar in the ranking order of performance over the set of cultivated *B. oleracea* tested.

Whereas specialist herbivores are adapted to specific chemicals of their host plant, performance of generalists is typically affected by high levels of these chemical defences (van der Meijden 1996; Schoonhoven et al. 2005). The 3-fold higher amount of GS in the native *B. nigra* indeed resulted in poor performance of the generalist *M. brassicae* and matched earlier findings of a negative correlation between performance and foliar concentration of glucosinolates for generalists (Chew 1988; Giamoustaris and Mithen 1995; Li et al. 2000). The better performance of *P. rapae* and intermediate performance of *P. xylostella* on *B. nigra* matched the general pattern of tolerance of specialists to glucosinolates (Chew 1988; Bodnaryk 1997; Li et al. 2000; Kliebenstein et al. 2002; Müller and Sieling 2006; Gols et al. 2008a,b) or the stimulating effect on caterpillar feeding by these compounds (van Loon et al. 2002). However, within cultivated plants the performance of the three herbivore species did not correlate with the concentrations of total GS or any single GS compound, nor was the performance of *M. brassicae* the poorest on *B. nigra*.

Differences in herbivore performance could also not be correlated with glucosinolate concentrations measured after herbivory. After feeding by the three herbivores, indole glucosinolate concentrations were different for the herbivore species. *Mamestra brassicae* and *P. xylostella*, which exhibited a similar ranking order of performance on the eight cultivars, were different in their effect on indole glucosinolates. Depending on the type of herbivore feeding on the plant, indole GS concentrations had increased 2-4-fold after 6 days of herbivory. Indole GS concentrations in leaves of *Brassica* are known to be stable over crop development time (Fieldsend and Milford 1994), suggesting that the extent of the increases we found are due to induced response of the plant after herbivory in all three herbivore treatments rather than associated with plant age. The induction of indole

Table 2. F-test for repeated measurement Mixed model analysis of the concentration of glucosinolates in the plants, testing the factors cultivar, caterpillar species feeding on the plant and the time of measurement after herbivore feeding, as well as the factorial interactions. [1]P-values are significant at α = 0.05.

	Factor						Interaction							
	Cultivar (1) (d.f. = 8)		Caterpillar (2) (d.f. = 8)		Time (3) (d.f. = 1)		(1*2) (d.f. = 16)		(1*3) (d.f. = 8)		(2*3) (d.f. = 2)		(1*2* 3) (d.f. = 16)	
Compound	F	P	F	P	F	P	F	P	F	P	F	P	F	P
Alkenyl glucosinolates														
Glucoiberin (IBE)	21.68	<0.001[1]	1.24	0.29	0.12	0.74	1.22	0.25	0.34	0.95	0.18	0.83	0.63	0.86
Sinigrin (SIN)	59.43	<0.001[1]	1.90	0.15	0.27	0.60	2.72	<0.001[1]	0.84	0.57	1.76	0.18	1.35	0.17
Progoitrin (PRO)	5.15	<0.001[1]	0.54	0.58	10.17	0.002[1]	0.97	0.49	1.40	0.20	1.46	0.23	0.68	0.81
Glucoraphanin (RAPH)	16.03	<0.001[1]	1.77	0.17	2.64	0.11	0.87	0.61	0.24	0.98	0.32	0.73	0.26	0.99
Gluconapin (GNA)	13.41	<0.001[1]	0.79	0.46	4.35	0.04 [1]	1.28	0.28	0.23	0.99	0.44	0.64	0.35	0.99
Indolyl glucosinolates														
4-hydroxyglucobrassicin (4OHGBC)	9.28	<0.001[1]	13.29	<0.001[1]	140.10	<0.001[1]	2.11	0.009[1]	4.14	<0.001[1]	4.01	0.02[1]	1.08	0.38
Glucobrassicin (GBC)	13.41	<0.001[1]	7.92	<0.001[1]	1.03	0.31	2.29	0.004[1]	1.05	0.40	1.26	0.29	0.69	0.81
4methoxyglucobrassicin (MGBC)	8.22	<0.001[1]	9.98	<0.001[1]	33.54	<0.001[1]	1.03	0.43	0.44	0.90	3.54	0.03[1]	0.14	0.99
Neo-glucobrassicin (NEOGBC)	15.74	<0.001[1]	11.34	<0.001[1]	1.52	0.22	1.05	0.41	1.12	0.35	0.13	0.88	0.38	0.99

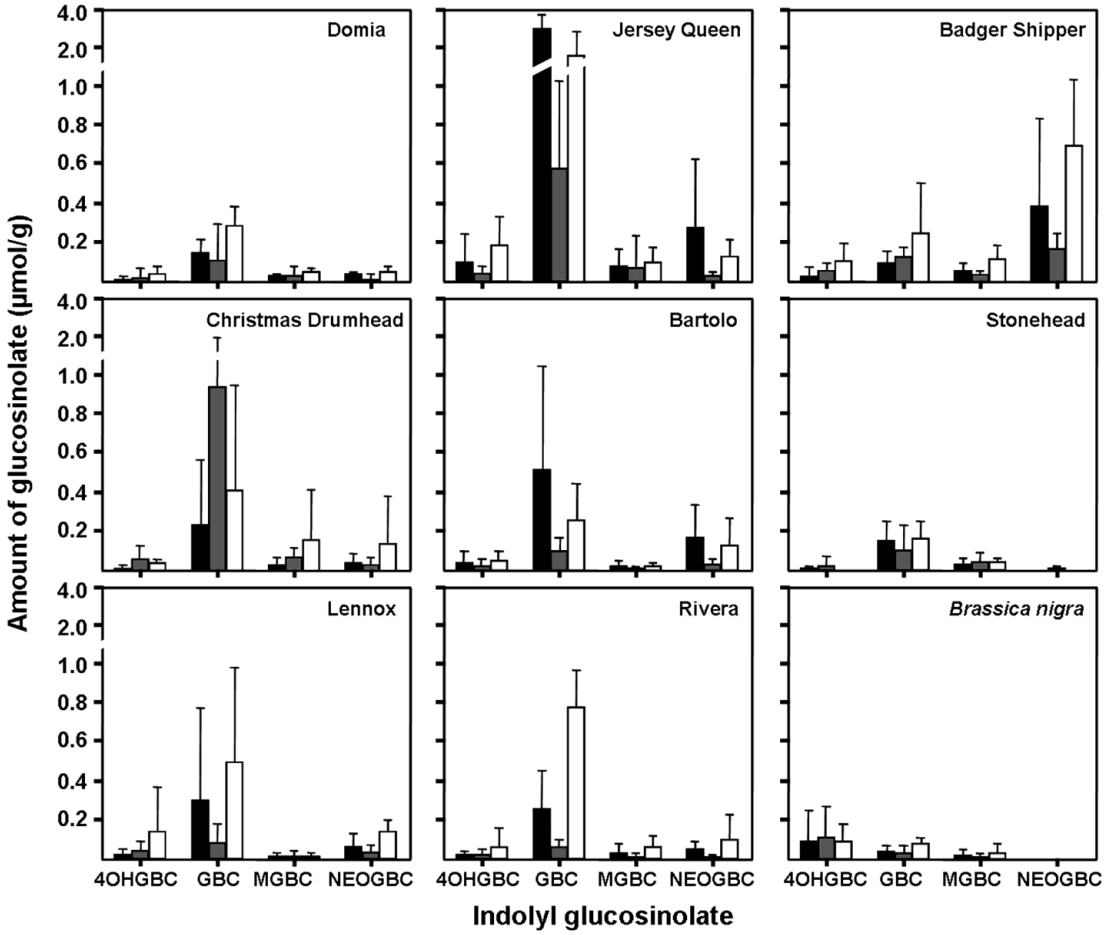

Figure 2. Indole glucosinolate content of eight *Brassica oleracea* cultivars and *Brassica nigra* after 6 days of feeding by different herbivores: *Pieris rapae* [(black bars (average + SEM)], *Plutella xylostella* (grey bars), and *Mamestra brassicae* (white bars). *Pieris rapae* and *M. brassicae* feeding resulted in significantly higher amounts of each indole glucosinolate compound than when *P. xylostella* was feeding on the plants (Mixed model, $P<0.001$).

GS has been related to biotic stress such as herbivory (Koritsas et al. 1991; Rostás et al. 2002; Traw and Dawson 2002; Mewis et al. 2006; Kim and Jander 2007) or plant hormones that regulate biotic stress responses (Bodnaryk 1994; Loivamäki et al. 2004). As hypothesized, feeding by the generalist *M. brassicae* resulted in higher concentrations of indole GS than feeding by the specialist *P. xylostella*. However, the other specialist (*P. rapae*) elicited a similar response as *M. brassicae*, making the degree of host plant specialization not a plausible explanation for differential response of defence expression in plants after herbivory. The suggested differential induction of indole GS may have resulted from herbivore differences in the amount and pattern of damage they inflicted, as well as differences in constituents of oral secretions (Mattiaci et al. 1995). The first larval stages of *P. xylostella* are mining; due to their much smaller size than *M. brassicae* or *P. rapae* caterpillars, the later stages of *P. xylostella* cause less damage, distributed over many small holes (Olsson and Jonasson, 1994). The observed similarity in effect on indole GS for the

Table 3. Regression analysis of caterpillar mass of the three herbivores correlated to (A) the constitutive and (B) the induced concentration of foliar glucosinolates

A)	Constitutive glucosinolate content								
	Total glucosinolates			Alkenyl glucosinolates			Indolyl glucosinolates		
Herbivore species	R	F	P	R	F	P	R	F	P
Pieris rapae	0.62	4.33	0.08	0.60	4.01	0.09	0.42	1.50	0.26
Plutella xylostella	0.37	1.11	0.33	0.37	1.10	0.33	0.11	0.08	0.78
Mamestra brassicae	-0.13	0.12	0.74	-0.13	0.12	0.74	0.02	0.00	0.96
B)	Induced glucosinolate content								
	Total glucosinolates			Alkenyl glucosinolates			Indolyl glucosinolates		
Herbivore species	R	F	P	R	F	P	R	F	P
Pieris rapae	0.53	2.70	0.14	0.46	1.83	0.22	0.25	0.48	0.51
Plutella xylostella	0.23	0.39	0.55	0.23	0.37	0.56	0.01	0.00	0.98
Mamestra brassicae	-0.17	0.20	0.67	-0.21	0.32	0.59	0.32	0.79	0.40

two categories of host plant specialization may be caused by similarity in feeding behaviour of *M. brassicae* and *P. rapae* caterpillars.

Although herbivores differentially affected the concentration of indole GS, the effectiveness of indole GS as chemical defence compound against caterpillars may be weak; interaction of myrosinase with indole GS results in only small amounts of toxic isothiocyanates that are unstable in biological fluids (Bones and Rossiter 2006; N Agerbirk pers. comm.). However, recent studies revealed that intact indole GS compounds deter aphids and negatively affect aphid reproduction even in the absence of myrosinase (Kim and Jander 2007). Breakdown of unstable indole GS in the absence of myrosinase results in yet unknown compounds (Kim and Jander 2007), which may also be effective against other herbivores. Other defensive chemicals, differences in primary metabolites serving as nutrients, and synergistic effects between compounds may explain similarity in performance across host plant specialization as found in this study. Herbivores, both specialists and generalists, are affected by enhanced nutrient levels in cultivars that resulted from artificial selection (Benrey et al. 1998; Fahey et al. 2001; Schoonhoven et al. 2005). Furthermore, cultivated *Brassica* plants were found to have higher levels of proteinase inhibitors than their native congeners (Broadway 1989) and these compounds were found to affect both generalist and specialist herbivores negatively (Broadway and Colvin, 1992). Micro-array studies revealed that the expression of genes coding for proteinase inhibitors in response to *P. rapae* feeding was higher in cv. Rivera than in Christmas Drumhead (Broekgaarden et al. 2007) and may account for poor performance of all three herbivores on cv. Rivera. Other physical traits such as thickness of the epicuticular wax layer may have reduced the performance of some species (Picoaga et al. 2003). The difference in performance between the two specialists may further be explained by a difference in resistance mechanism against toxins in *P. rapae* and *P. xylostella* (Agrawal 2000; van Dam et al. 2000; Agrawal and Kurashige 2003). *Plutella xylostella* is known to desulfate GS and may thereby prevent that the breakdown of GS by myrosinase results in highly toxic compounds (Ratzka et al. 2002), whereas *P. rapae* redirects the myrosinase-catalyzed GS hydrolysis to form nitriles instead of the more toxic isothiocyanates (Wittstock et al. 2004). Both detoxification strategies may result in different amounts of breakdown products of GS that can negatively affect performance of specialists (Li et al. 2000; Agrawal and Kurashige 2003). Despite the lack of a clear correlation between performance and amount of a single type of secondary

chemicals, i.e., GS, we have shown that the set of defence traits of plants may affect performance of two herbivores similarly across different degrees of host plant specialization and that this similarity may also occur for plant responses after herbivory.

Although the difference in indole GS expression found in this study did not correlate with larval performance of herbivores, these compounds may still function as enhanced defence when reducing oviposition acceptance of plants by herbivores. Glucosinolates have been found to reduce the acceptance of brassicaceous plants by generalist butterflies (Mithen et al. 1995), but on the other hand act as oviposition cues for specialist butterflies (van Loon et al. 1992; Giamoustaris and Mithen 1995; Moyes et al. 2000; Renwick et al. 2006; Bruinsma et al. 2007). Future studies on plant responses to herbivory in terms of glucosinolate content should therefore address the role of indole GS as directed against subsequent attackers or natural enemies of attackers to further elucidate the effects of inducible indole GS.

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Chapter 3

Picture: Erik Poelman

Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores

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Submitted

Abstract

Intraspecific variation in plants has been identified to play a major role in the composition and diversity of the associated insect community. Resistance traits of plants are likely candidates for mediating community composition. However, there is debate over whether total concentrations of chemical compounds or specific compounds determine herbivore resistance and how chemical diversity among plant genotypes in turn affects the composition of the associated herbivore community.
To study the role of specific chemical compounds in affecting the herbivore community, we used cultivated *Brassica oleracea*. The cultivars differ qualitatively in their glucosinolate profile, i.e. foliar composition of different glucosinolate compounds, and only little in the total concentration of glucosinolates, the secondary metabolites specific for the Brassicaceae family. In field and laboratory experiments, we tested whether individual compounds explained differences in herbivore community composition and whether herbivores with similar degree of host plant specialization covaried in their responses to variation in glucosinolate profiles.
In the field *Brassica oleracea* cultivars differed widely in abundance, species richness and composition of the herbivore community they harbored. Plants with high concentrations of the short side chain alkenyl glucosinolate, glucoiberin, harboured low herbivore diversity. Higher biodiversity was found when plants had glucosinolate profiles containing high concentrations of glucosinolates with elongated side chains, which are biosynthetically linked to glucoiberin. Although glucosinolates are known to have differential effects on generalist and specialist herbivores, all herbivores covaried in the direction of response to the intraspecific variation in foliar glucosinolate profiles of the *B. oleracea* cultivars. This observation is supported by the similarity in oviposition preference of the specialist *Pieris rapae* and generalist herbivore *Mamestra brassicae* in our laboratory studies using the same cultivars and this is due to the relatively low concentrations of glucosinolates in cultivars. Our results show that variation in the concentration of short side-chain glucosinolates affects the composition of the herbivore community associated with brassicaceous plants.

Key words: biodiversity, species richness, glucosinolates, direct defence, Shannon-Wiener Index, *Pieris rapae*, *Mamestra brassicae*

Introduction

A central issue in ecology is to understand the interactions between organisms within communities. This understanding is of even greater importance since it has been established that human-driven changes in the environment diminish the planet's biodiversity, disrupt species interactions and ecosystem functioning (Tilman 1999 and references therein). Plant -insect associations comprise most of the interactions in terrestrial ecosystems and make up a major share of the biodiversity of agro-ecosystems that nowadays cover a substantial part of our planet (Groombridge 1992). The study of these associations has played an important role in understanding ecological and evolutionary processes that underlie community biodiversity (Whitham et al. 2006). Not only is insect biodiversity shaped directly by the diversity of plants or their spatial distribution over the landscape (Root 1973; Di Giulio et al. 2001; Tscharntke and Brandl 2004), insect diversity is also strongly affected by intraspecific variation in plants (Fritz and Price 1988; Maddox and Root 1990). This effect of intraspecific variation at the first trophic level may even scale up to the fourth trophic level in insect communities (Bukovinszky et al. 2008) and extend to vertebrate predators (Bailey et al. 2006; Gruner and Taylor 2006). Therefore, variation in plant traits may significantly influence ecosystem biodiversity (Dungey et al. 2000; Hochwender and Fritz 2004; Wimp et al. 2005). Plant traits responsible for effects on higher trophic level biodiversity have been identified in a few cases only and include plant morphology and phytochemistry (Dungey et al. 2000; Johnson and Agrawal 2005; Bailey et al. 2006; Bangert et al. 2006a). Variation in phytochemistry of plant genotypes may include quantitative or qualitative differences in only a few chemical compounds and phytochemical composition may be heritable (van Dam and Vrieling 1994; Nielsen 1997; van Leur et al. 2006).

Intraspecific differences in foliar chemical profiles, i.e. the qualitative composition of the mix of phytochemicals, may have extensive consequences for ecosystem biodiversity and especially affect species that are in close reciprocal interaction with plants such as herbivorous insects (Johnson and Agrawal 2005). These compounds include plant family-specific secondary metabolites that may differentially determine host plant acceptance by herbivores. In the Brassicaceae, for example, high concentrations of glucosinolates and their breakdown products deter generalist herbivores, whereas specialists exploit these compounds in host plant selection (Chew 1988; Giamoustaris and Mithen 1995; Agrawal 2000). Although glucosinolates may also negatively affect larval performance of specialist herbivores (Agrawal and Kurashige 2003), the toxins are often found to function as oviposition cue or feeding stimulant for specialist herbivores (David and Gardiner 1966; van Loon et al. 1992, 2002; Renwick 2002). Total concentration of glucosinolates of a plant may, therefore, influence the rate of attack by specialist and generalist herbivores in opposite directions. However, the glucosinolates comprise a considerable molecular diversity (Fahey et al. 2001), and intraspecific natural variation in foliar glucosinolate profiles of brassicaceous species has been extensively reported (Mithen et al. 1995; Moyes et al. 2000; Kliebenstein et al. 2002; Gols et al. 2008a, b). When plant tissue is damaged, glucosinolates are hydrolyzed by myrosinases, yielding a variety of products such as isothiocyanates, thiocyanates, nitriles, epithionitriles and oxazolidines (Bones and Rossiter 2006). Among other factors, the type of glucosinolate determines the composition of enzymatically formed products that differ in toxicity for herbivores (Bones and Rossiter 2006).

It has been hypothesized that well-defended plants have a large number of different compounds so that they are more likely to possess defence traits against the whole suite of attacking herbivores (Jones and Firn 1991). Plants seem not to be constrained in maintainance of this diversity (Koricheva et al. 2004), although compound levels may be negatively correlated when one compound is a biosynthetic precursor for the other (Kroymann

et al. 2001). When these compounds differ in the extent to which they mediate host plant acceptance by herbivores, there are trade-offs between maintaining specific compounds or converting these into others, depending on the degree of host plant specialization of the attacker (Jaenike 1990; van der Meijden 1996). The debate whether specific compounds in plant defence profiles determine herbivore resistance remains unresolved. Furthermore, it is even less studied how qualitative differences in phytochemistry in turn affect the composition of the associated herbivore community.

Here we address whether differences in foliar glucosinolate profiles in *Brassica oleracea* affect insect herbivore biodiversity and the composition of the herbivore community in the field. As a model for intraspecific phytochemical variation we used four cultivars of *B. oleracea* that differ in glucosinolate profiles (Poelman et al. 2008c) and gene transcription profiles in response to herbivory (Broekgaarden et al. 2007). Their glucosinolates all consist of a ß-thio-glucose moiety, but have a variable side chain (Bones and Rossiter 2006). Alkenyl-glucosinolates have a side chain that consists of a methylene chain of variable length that is controlled by genes encoding methylthioalkylmalate synthase (MAM) (Kroymann et al. 2001). Transcription of MAM-like genes results in elongation of glucosinolate side chains from 3 carbon atoms (C3) to C4 and up to C8, thereby affecting the ratio of, for example, C3 to C4 compounds in the glucosinolate profile (Kroymann et al. 2001; Field et al. 2004). The history of cultivation of *B. oleracea* has resulted in a decrease in total glucosinolate levels compared to wild type plants (Harvey et al. 2007; Gols et al. 2008a, b). However, at the same time cultivation has led to a diversity of chemical profiles with cultivars having similar total concentrations of glucosinolates but different mixtures of C3 and C4 compounds in their profile. This variation can be readily used to test the effect of different chemical profiles that are within the range of those found within the natural variation of *B. oleracea* (Mithen et al. 1995; Moyes et al. 2000).

In the field we tested the hypotheses that 1) specific compounds affect herbivore biodiversity and 2) herbivores with similar degree of host plant specialization covary in their response to variation in glucosinolate profiles. To test whether herbivore abundance and species composition on the different cultivars in the field correspond with their host plant preference and is not a result of apparent competition between herbivores or differences in predation rates, we conducted additional laboratory experiments. We selected a specialist (*Pieris rapae*) and a generalist herbivore (*Mamestra brassicae*). Both species were recorded on the cultivars in the field. By choosing a specialist and a generalist, we could analyze whether herbivores with different degree of host plant specialization differ in their response to intraspecific variation in phytochemistry. Finally, we discuss the ecological implications of variation in glucosinolate profiles for ecosystem biodiversity.

Methods

Plants and insects

We used the following white cabbage cultivars (*Brassica oleracea* var. *alba* L.; donors are given in brackets): Badger Shipper, Christmas Drumhead (Centre for Genetic Resources, CGN-Wageningen, The Netherlands), Lennox and Rivera (Bejo Zaden BV, Warmenhuizen, The Netherlands). Additionally, we used *Brassica nigra* L. to confirm whether herbivores observed on *B. oleracea* were also present on a wild congener and, thus, whether our model system harboured a natural herbivore community. Because of its different phenology and life history compared to *B. oleracea*, we excluded *B. nigra* from all comparative analyses. Seeds of *B. nigra* were collected from a population north-west of Wageningen, The Netherlands and propagated by open pollination several times since. Seeds of all plants were germinated on peat soil (Lentse potgrond, No. 4) and seeds for the

common garden experiment were directly sown into peat soil cubes. Trays with soil cubes containing three-week-old seedlings were placed outside during the day to adapt plants to field conditions. The plants were transplanted with their soil cubes to the soil at the experimental site when they were five weeks old. Plants used in greenhouse experiments were transferred as two-week-old seedlings to 1.45 L pots containing the same potting soil. Pots were placed in a greenhouse, providing the plants with a 16/8 (light/dark) photoperiod with SON-T light (500 µmol/m^2/sec) in addition to daylight, at 18ºC–26ºC and 40-70% relative humidity. When the plants were four weeks old, they were fertilized weekly by applying 100 ml nutrient solution (KristalonTM®, concentration 3 g/l, (16N: 6P: 20K: 3Mg)) to the soil. We used seven-week-old plants that had 12 true leaves for herbivore preference and performance experiments.

The two Lepidoptera species studied in preference and performance experiments, *Pieris rapae* L. (Pieridae) and *Mamestra brassicae* L. (Noctuidae), originated from the respective cultures maintained at the Laboratory of Entomology, Wageningen University. The host-plant used for rearing was Brussels sprouts (*Brassica oleracea* var. *gemmifera* L. cultivar Cyrus). Cultures were kept in a climatized room at 20-22ºC, 50-70% relative humidity and a 16/8 hour light/dark photoperiod. *Mamestra brassicae* moths were offered filter paper as oviposition substrate, without contact to cabbage plants.

Common garden experiment

To assess how direct defence of field-grown plants affected their associated herbivore biodiversity in the field, we established a common garden experiment in an agricultural field in the vicinity of Wageningen, The Netherlands. Forty plots (6 x 6 m), each planted with one of the four cultivars or *Brassica nigra* were established, according to a randomized design. Five-week-old seedlings rooted in their soil cubes were transplanted to the field in week 19 (9 May) of 2005. We planted 49 plants in a square of 7 x 7 plants per plot with spacing of 75 cm between plants. Plots were isolated from each other by a strip of 6 m that was sown with a grass mixture of *Lolium* and *Poa* species. From week 23 (6 June) until week 37 (16 September), the central nine plants of each plot were surveyed weekly for the presence of naturally colonizing herbivorous insects. Each plant was surveyed by investigating both sides of all its leaves. We found eleven species of herbivorous insects that have all been reported previously to be affiliated with *Brassica* plants (Root 1973) (Table 1). Of these eleven herbivores, we could not accurately count the number of whiteflies and thrips without damaging the plants. These herbivores were therefore excluded from further analy-

Table 1. Herbivore species found on *Brassica oleracea* cultivars and their degree of host plant specialization.

Species	Order	Family	Feeding type	Host specificity
Pieris rapae	Lepidoptera	Pieridae	Leaf chewer	Specialist
Pieris brassicae	Lepidoptera	Pieridae	Leaf chewer	Specialist
Plutella xylostella	Lepidoptera	Yponomeutidae	Leaf chewer	Specialist
Mamestra brassicae	Lepidoptera	Noctuidae	Leaf chewer	Generalist
Autographa gamma	Lepidoptera	Noctuidae	Leaf chewer	Generalist
Brevicoryne brassicae	Hemiptera	Aphididae	Phloem feeder	Specialist
Myzus persicae	Hemiptera	Aphididae	Phloem feeder	Generalist
Phyllotreta undulata	Coleoptera	Chrysomelidae	Leaf chewer	Specialist
Phyllotreta atra	Coleoptera	Chrysomelidae	Leaf chewer	Specialist
Aleyrodes proletella	Hemiptera	Aleyrodidae	Phloem feeder	Specialist
Thrips tabaci	Thysanoptera	Thripidae	Cell content feeder	Generalist

ses. For each week, the number of individuals per species counted on the nine plants of a plot was averaged. These values were used to calculate at plot level: a) the total abundance of herbivores, b) their species richness; and two indices of biodiversity: c) the Shannon-Wiener Index (H`), and d) the Simpson's diversity Index (1-D). The latter two biodiversity indices describe herbivore diversity by incorporating both the richness of species as well as the evenness of their distribution. The Simpsons Index of diversity (D) presents the chance that random draws of two individuals from a plot represent individuals of the same species. The lower the value of D, the higher is the diversity of the sample. 1-D is therefore often presented such that higher values correspond with higher diversity. The more abundant species receive a higher weight based on this index than rare species and the index thus estimates evenness of a sample. The Shannon-Wiener index also takes into account the species richness and the abundance of each species, but does not give an interpretation in terms of chance of draws from a population. Both unique species and higher evenness increase the value. Both indices are the most commonly used indices to describe biodiversity.

Glucosinolate composition of field plants

The foliar glucosinolate content of each cultivar was quantified at plot level, five weeks after we started the herbivore survey. In week 28, we collected two leaf disks of a total of five plants for each plot, using a cork borer (diameter of 2.3 cm). The ten leaf disks were pooled per plot and stored on ice. Within two hours after sampling the first plant, the leaf disks were transferred to a -80 ºC freezer. The frozen samples were freeze-dried and ground to a fine powder. Amounts of 100 mg of ground leaf material per sample were dissolved in methanol. The extract was desulphatased on a DEAE-Sephadex A25 column and the glucosinolate content was assessed by high performance liquid chromatography (HPLC), using the method described by van Dam et al. (2004). Glucosinolate detection was performed with a photodiode array (PDA) detector (200 – 350 nm) with 229 nm as the integration wavelength. A sinigrin (sinigrin monohydrate, Sigma, St. Louis, MO, USA) concentration series was used as an external standard. We used the correction factors at 229 nm from Buchner (1987) and the EC (EC 1990) to calculate the concentrations of the glucosinolates. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with standards kindly provided by M. Reichelt, Max Planck Institute for Chemical Ecology, Jena, Germany and a certified rapeseed standard (Community Bureau of Reference, Brussels, code BCR-367R).

Preference and performance of P. rapae and M. brassicae

Two of the Lepidoptera species found in the field that had a different degree of host plant specialization, the specialist *Pieris rapae* (Pieridae) and the generalist *Mamestra brassicae* (Noctuidae), were studied in detail for their response to the four cultivars. We present their population development in the field and quantified their oviposition preference and larval performance in the laboratory. Population development of these species was constructed from the data obtained in the common garden experiment and revealed that both species are present in similar numbers on cultivars Rivera and Lennox. We therefore did not include Lennox in the preference and performance experiments.

Growth rate, as a correlate of performance of *P. rapae* and *M. brassicae* caterpillars, was measured by placing single neonate caterpillars in Petri dishes and feeding them *ad libitum* with leaf material of Badger Shipper, Christmas Drumhead or Rivera. Every third day, the caterpillars were weighed on a microgram balance and every other day the leaf material was replaced with fresh leaves of the same plant. The Petri dishes were

placed in a climate cabinet at 22 ± 2 ºC, 60 ± 10 % r.h., and a 16/8 hour light/dark photoperiod.

Host plant preference of *P. rapae* butterflies was tested in a three-choice situation between a plant of cultivar Badger Shipper, Christmas Drumhead and Rivera. Freshly eclosed butterflies were placed in a cage where they were provided with a 10% sucrose solution and allowed to mate. Two days later, pairs of *P. rapae* were transferred to the experimental oviposition cages (67x50x75 cm; one male and one female butterfly per cage) that contained the three-choice situation in the same greenhouse compartment (22 ± 2 ºC, 60 ± 10 % r.h., 16/8 hour light/dark photoperiod). During the experiment the oviposition cages were illuminated by SON-T, 500W sodium vapor lamps, in addition to natural daylight. Butterflies were allowed to oviposit on the plants from 9:00 A.M. to 2:00 P.M. Thereafter plants were removed and eggs were counted. We repeated the experiment 14 times and established that the cultivars Badger Shipper and Rivera differed most in host plant acceptability for *P. rapae*.

The experimental setup used for *P. rapae* proved to be unsuitable for testing host plant preference of *M. brassicae* females that laid most of their egg clutches on the wood of the cage. We therefore used plastic cages (22 cm height, 13 cm diameter (Poelman et al. 2008b)) and studied the responses to the cultivars identified as extremes in the tests for *P. rapae*. For *M. brassicae*, we conducted 30 replicates of two-choice situations in plastic cages placed in a climatized room (22 ± 2 ºC, 60 ± 10 % r.h., 16/8 hour light/dark photoperiod) by offering a choice between an excised leaf of the cultivars Badger Shipper and Rivera. Excised leaves of seven-week-old greenhouse plants were placed in glass vials containing tap water to keep the leaves turgid. After 24 h, leaves were removed and the numbers of egg batches as well as the numbers of eggs were counted.

Statistical analysis

We used repeated measurements mixed models to determine whether plants with different qualitative glucosinolate profiles harboured different herbivore communities. At the plot level, the abundance of *P. rapae*, *M. brassicae*, total number of herbivores, species richness, the Shannon-Wiener Index (H`) and the Simpson's diversity Index (1-D) were each modeled using the Proc Mixed procedure with repeated structure type AR(1) of SAS version 9.1. In each model we included the factors cultivar, time and their interaction. We specifically addressed the question whether differences in specific chemical compounds between cultivars could explain differences in herbivore biodiversity. We correlated phytochemistry assessed at the plot level with biodiversity in the study plots. To describe the most important qualitative differences in glucosinolate profiles between cultivars, we used principal component analysis (PCA) to analyze the multivariate chemical data, comprised of ten glucosinolate compounds found in the cultivars. The first PC axis explained 72% of

Table 2. Cultivar and time effects on herbivore abundance, species richness, Shannon Wiener diversity Index (H`), Simpson's diversity Index (1-D) and development of populations of two Lepidoptera species. For each factor we calculated *F* statistics in Mixed models. Boldface type present significant effects at $P < 0.05$.

	Abundance		Richness		Shannon-Wiener index		Simpson's diversity index		*P. rapae* abundance		*M. brassicae* abundance	
Factor	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Cultivar	44.21	**<0.001**	28.94	**<0.001**	42.41	**<0.001**	29.30	**<0.001**	3.63	**0.025**	5.86	**0.003**
Time	124.41	**<0.001**	53.46	**<0.001**	15.39	**<0.001**	9.85	**<0.001**	81.59	**<0.001**	30.40	**<0.001**
C x T	3.05	**<0.001**	1.59	**<0.001**	2.66	**<0.001**	2.06	**<0.001**	0.95	0.56	1.93	**0.001**

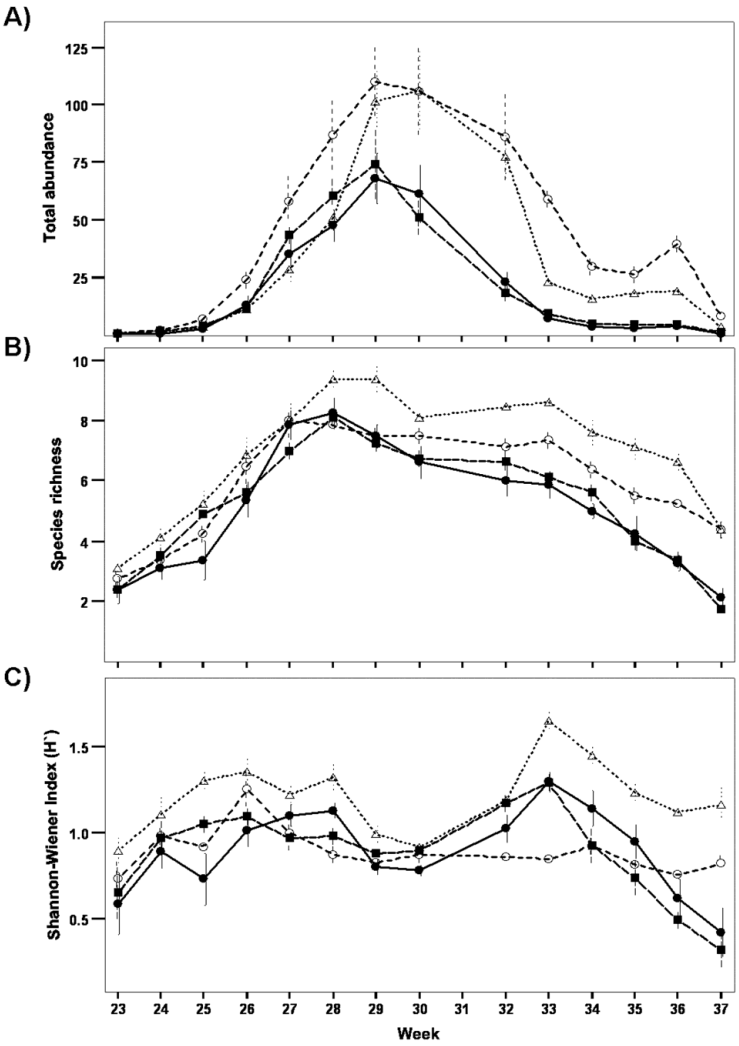

Figure 1. Cultivar differences in (A) herbivore abundance (number of individuals), (B) species richness (number of species) and (C) Shannon-Wiener Index (H`) of herbivore communities in the field (average and SE). Cultivars: Badger Shipper (open triangle), Christmas Drumhead (open circle), Lennox (closed square), Rivera (closed circle).

the variation in glucosinolate content with an eigenvalue of 6.47 and correlated with an increase in concentration of glucoiberin and a decrease of glucoraphanin (loading scores of glucoiberin: 0.93 and glucoraphanin: -0.19 on PC1). Other PC axes had eigenvalues lower than 1 and explained less than 10% of the total variation. The scores of each field plot on the first axis were used in Generalized Linear Models as a co-variate to test for a correlation with herbivore abundance, richness and biodiversity. Cultivar identity was included as a fixed factor into these models. We included the interaction between cultivar and glucosinolate-PC score into the model to test for differences between cultivars in relationship between glucosinolates and diversity parameters. Per plot, the scores for herbivore abundance, richness and biodiversity are averaged values over the 15 study weeks.

To determine which species in the herbivore communities were affected most by cultivar differences, we constructed principal response curves (PRC) using the CANOCO software package 4.51 (Ter Braak 1988). The PRC method uses partial redundancy analysis (RDA) and plots the first principal component of the treatment effect against time by contrasting each treatment against a preset control. We set the cultivar Rivera as a control

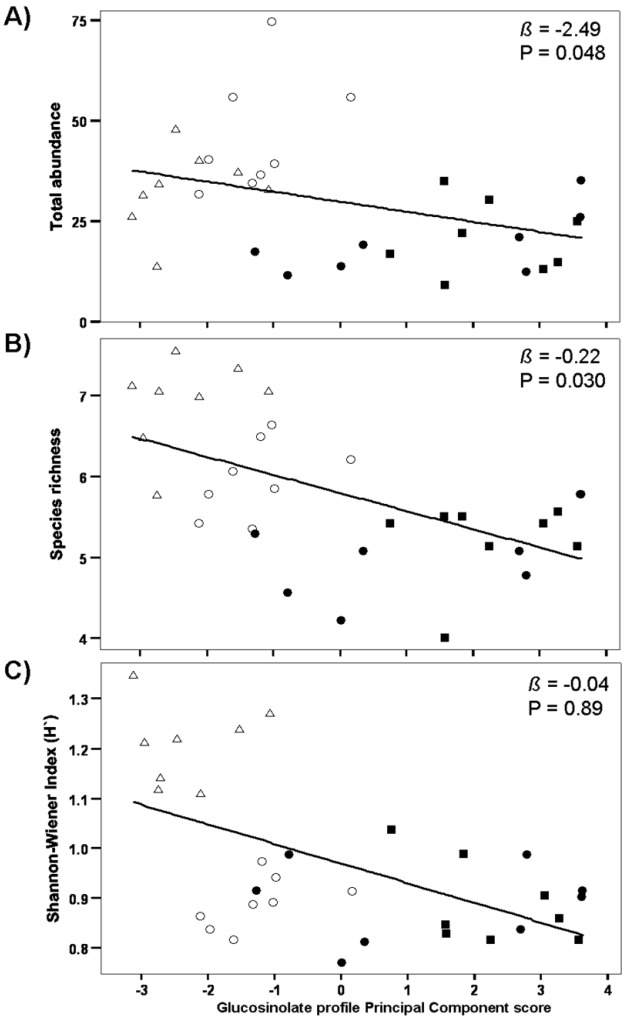

Figure 2. Correlations between the scores of each plot on the glucosinolate first principal component and (A) total herbivore abundance, (B) species richness, (C) Shannon-Wiener Index of biodiversity of field plots. The principal component explains 72% of the variation in glucosinolate content of the four *B. oleracea* cultivars (Badger Shipper (open triangle), Christmas Drumhead (open circle), Lennox (closed square), Rivera (closed circle) and correlates with increased concentrations of the glucosinolate glucoiberin and decreased concentration of glucoraphanin.

and, thus, the vertical axis of the PRC diagram shows the contrast of the other three cultivars with Rivera. The PRC method is constrained and extracts information only from the part of the variance that is explained by the environmental factor (cultivar in our case) and implements time (weeks in our case) as a co-variable. Since high abundance values influence the result of the PRC analysis stronger than low abundance values, we log-transformed the species abundance scores. To test for significance of the principal component we used Monte Carlo permutation tests. The reported P-value is based on 999 permutations. Associated with the PRC diagram we present the set of species weights on the first principal component. The species weight describes the relative difference of abundance between cultivars for each herbivore species. A positive weight can be interpreted as a larger abundance of the particular species on the cultivar that also has a positive PRC score (Lepŝ and Šmilauer 2003). To test whether specialist and generalist herbivores differed in their response to the cultivars we first performed cluster analysis. Cluster analysis creates a dendrogram that depicts how herbivores covaried in their abundance over the field plots measured, with all the herbivore species individually plotted on the tips of the dendrogram.

Table 3. Glucosinolate profiles of four cultivars of *Brassica oleracea* and a wild population of *Brassica nigra* (μmol / g dry weight (SE)):
Alkenyl C3 glucosinolates: 3-methylsulfinylpropyl glucosinolate (glucoiberin (IBE)), 2-propenyl glucosinolate (sinigrin (SIN)), R-2-hydroxy-3-butenyl glucosinolate (progoitrin (PRO));
Alkenyl C4 glucosinolates: 4-methylsulfinylbutyl glucosinolate (glucoraphanin (RAPH))
Alkenyl C5 glucosinolates 4-pentenyl glucosinolate (glucobrassicanapin (GBN))
Indolyl glucosinolates: 4-hydroxy-3-indolylmethyl glucosinolate (4-hydroxyglucobrassicin (4OHGBC)), 3-indolylmethyl glucosinolate (glucobrassicin (GBC)), 4-methoxy-3-indolylmethyl glucosinolate (4-methoxyglucobrassicin (MGBC)), 1-methoxy-3-indolylmethyl glucosinolate (Neo-glucobrassicin (NEOGBC)).

	IBE	SIN	PRO	RAPH	4OHGBC	GBN	GBC	MGBC	NEOGBC	Total
Brassica oleracea										
Badger Shipper	1.35 (0.26)	0.11 (0.05)	0.09 (0.01)	1.74 (0.23)	0.03 (0.01)	0.07 (0.01)	0.84 (0.11)	0.03 (0.00)	0.90 (0.15)	5.14 (0.62)
Christmas Drumhead	1.87 (0.22)	0.77 (0.34)	0.20 (0.04)	1.48 (0.30)	0.02 (0.01)	0.13 (0.01)	2.21 (0.24)	0.04 (0.00)	0.04 (0.01)	6.77 (0.70)
Lennox	5.64 (0.38)	0.67 (0.12)	0.06 (0.01)	0.45 (0.04)	0.02 (0.01)	0.10 (0.01)	1.54 (0.33)	0.03 (0.00)	0.06 (0.01)	8.56 (0.60)
Rivera	4.52 (0.60)	0.63 (0.12)	0.08 (0.02)	0.54 (0.09)	0.01 (0.02)	0.12 (0.02)	2.30 (0.62)	0.03 (0.01)	0.12 (0.03)	8.36 (1.47)
Brassica nigra	0.07 (0.05)	28.95 (2.60)	-	0.09 (0.06)	-	0.01 (0.01)	0.12 (0.05)	-	0.01 (0.01)	29.25 (2.62)

The species that link closest with each other on the dendrogram covary strongest in their abundance across field plots. To perform the analyses, we calculated the mean abundance of each herbivore for each of the field plots that were monitored over 15 weeks. The abundance scores were standardized so that the abundance of each herbivore species had a mean of zero and a standard deviation of one. The standardized abundance values of each species per field plot were then used in the calculation of the Pearson's correlation matrix (Appendix A). We used the Ward's linkage method to determine the linkage and distance between herbivore species. To statistically test whether herbivores with the same host plant range correlated more strongly in their abundance distribution than species with different host plant range, we used ANOVA tests on the Pearson's correlation scores. We tested whether r-values within generalist and specialist herbivores were higher than r-values between these two herbivore groups.

The laboratory experiments to measure preference and performance were analyzed with the statistical programme SPSS 12.0. We used G-statistics to analyze the oviposition preference of *P. rapae* in choice tests consisting of three cultivars. An oviposition preference of *M. brassicae* in two-choice tests was analyzed by applying Wilcoxon matched-pair signed-ranks tests for both the number of egg batches and number of eggs. The effect of cultivar on growth rate of caterpillars of both species was analyzed with repeated measurements ANOVA for the log-normalized weight of caterpillars.

Results

In the field we found eleven herbivore species (Table 1) that were all attacking the wild congener *B. nigra* as well as the four cultivars of *B. oleracea*. Although most Lepidoptera species were present in much lower numbers on *B. nigra*, our model system of cultivated plants harboured an herbivore community comparable to congeneric wild type plants. Most of the herbivore species that we recorded in our plots were found to have two or more generations over the study period. Synchronization of generations between different herbivore species resulted in highest species richness and herbivore abundance during the study weeks 28 and 29 (Figure 1). Cultivars of *B. oleracea* differed substantially in the total abundance of herbivores, species richness, Shannon-Wiener index (H`) and Simpson's index of diversity (1-D) (Figure 1, Table 2). Cultivar Badger Shipper harboured high herbivore abundance, was richest in species and had the highest herbivore biodiversity. Cultivar Christmas Drumhead was similar to Badger Shipper in herbivore abundance, had intermediate species richness, but relatively low biodiversity. This was a result of the susceptibility of this cultivar to a particular herbivore species, the cabbage aphid *Brevicoryne brassicae*, which reached a high population size on Christmas Drumhead compared to Rivera and Lennox (Broekgaarden et al. 2008a). The cultivars Rivera and Lennox did not differ significantly for any of the herbivore diversity parameters (Figure 1). Both cultivars were characterized by having low numbers of herbivores and fewer herbivore species per plant (Figure 1), although all herbivore species were present on these cultivars.

To address the question that the foliar concentration of specific glucosinolate compounds of the cultivars could be responsible for the difference in herbivore biodiversity and not the total glucosinolate concentration that is relatively low as a result of directional selection by humans, we measured the foliar glucosinolate content under field conditions at the peak of herbivore diversity (week 28). As expected, cultivars differed more in the composition of their glucosinolate profile than in the total concentration of foliar glucosinolates (Table 3). Total glucosinolate concentration did not significantly affect herbivore abundance, richness or biodiversity (GLM, d.f. = 1, abundance: $\chi2 = 2.97$, $P = 0.09$; richness: $\chi2 = 3.18$, $P = 0.08$; Shannon-Wiener Index: $\chi2 = 0.40$, $P = 0.53$). PCA analysis on the

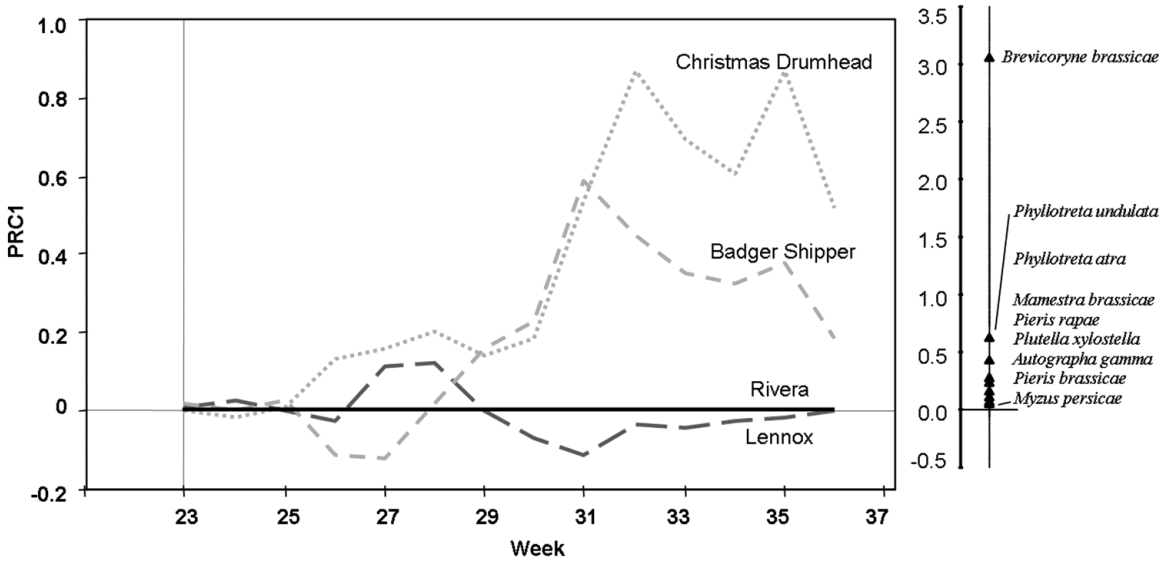

Figure 3. Principal Response Curve (PRC) for herbivore species abundance over time for four cultivars of *B. oleracea*. Cultivar differences are related to the control cultivar Rivera (black continuous line). Species weights on the first principal component are depicted in a score plot on the right side of the figure. Cultivar Lennox (striped black line) is similar in species composition to the control Rivera. The cultivars Christmas Drumhead (striped grey line) and Badger Shipper (stippled grey line) have higher abundance of all herbivore species.

glucosinolate profiles of plants identified that the concentration of glucoiberin (3-methylsulfinylpropylglucosinolate) that has a C3 side chain, negatively correlated with the concentration of glucoraphanin (4-methylsulfinylbutylglucosinolate), that has a C4 side chain. Rivera and Lennox had high concentrations of the short side chain compound, glucoiberin, whereas the concentration of this compound was lower in Christmas Drumhead and even more so in Badger Shipper. The negative association between the two compounds had a high loading on the first PC axis (glucoiberin: 0.93, glucoraphanin: -0.19) that explained 72% of the variation in foliar glucosinolate content of the cultivars. For all three biodiversity parameters we found that cultivars harboured a different degree of diversity (GLM, d.f. = 3, abundance: $\chi2 = 29.221$ $P < 0.001$; richness: $\chi2 = 31.772$ $P < 0.001$; Shannon-Wiener Index: $\chi2 = 16.192$, $P = 0.001$). The scores of field plots on the first phytochemical PC, included as a co-variate into the model, correlated negatively with the total herbivore abundance and species richness of the field plots (Figure 2) (GLM, d.f. = 1, abundance: $\chi2 = 3.904$, $P = 0.048$; richness: $\chi2 = 4.722$, $P = 0.03$). However, the glucosinolate profile of field plots did not significantly correlate with the Shannon-Wiener Index (GLM, d.f. = 1, $\chi2 = 0.018$, $P = 0.89$). The interaction between glucosinolate profiles and cultivars was not significant for each of the biodiversity parameters (GLM, d.f. = 3, abundance: $\chi2 = 2.610$, $P = 0.46$; richness: $\chi2 = 0.777$, $P = 0.86$; Shannon-Wiener Index: $\chi2 = 4.771$, $P = 0.19$). This means that indeed an increase of glucosinolate side chain length, rather than total glucosinolate concentration, was associated with a higher susceptibility to herbivores and consequently enhanced herbivore biodiversity on these plants.

Response by generalist versus specialist herbivores

To identify whether generalist or specialist herbivore species were responsible for the

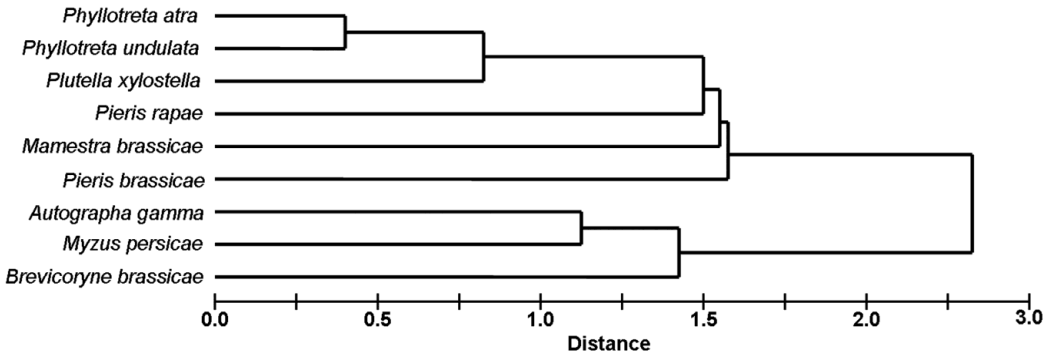

Figure 4. Dendrogram from cluster analysis using Ward's linkage method depicting the covariation in abundance of herbivorous insects among field plots of four *B. oleracea* cultivars. Species that link with the lowest distance scores are most similar in their distribution over the field plots.

lower abundance and biodiversity on Rivera and Lennox compared to Badger Shipper and Christmas Drumhead plants, we contrasted herbivore communities on cultivars by principal response curves (PRC). The PRC analysis identified that all herbivore species were more abundant on Badger Shipper and Christmas Drumhead compared to Rivera and Lennox (First RDA axis explained 9.6%, Monte Carlo permutation test $F = 193.398$, $P < 0.001$) (Figure 3), although the magnitude of the response differed per species. The loadings of herbivore species on the first RDA axis identified that the Cabbage aphid (*B. brassicae*) showed the largest difference in abundance between cultivars, whereas the other species were responding less strongly to cultivar differences. Cluster analysis for the covariation of herbivores in their abundance distribution over plots revealed that generalist and specialist herbivores did not significantly differ in their response to cultivars (ANOVA, d.f. = 1, $F = 2.955$, $P = 0.10$) (Figure 4, Appendix A).

Analysis of the population development of a specialist (*P. rapae*) and generalist (*M. brassicae*) herbivore in the field confirmed the similarity in response of the two types of herbivores. Both *P. rapae* and *M. brassicae* were more abundant on Badger Shipper and Christmas Drumhead than on Rivera or Lennox (Figure 5, Table 3). These differences correspond with differences in oviposition preference of adult butterflies and moths as recorded under controlled laboratory conditions (Figure 6). *Pieris rapae* and *M. brassicae* both preferred Badger Shipper plants over other cultivars (*P. rapae*: G-statistics, $\chi^2 = 80.24$ $P < 0.001$; *M. brassicae*: Wilcoxon matched-pair signed-ranks test, egg batches: $Z = -2.230$, $P = 0.019$; eggs: $Z = -2.983$, $P = 0.003$). Badger Shipper plants also sustained higher performance of caterpillars of both herbivores than other cultivars (repeated measurements ANOVA, *P. rapae*: cultivar df = 2, $F = 8.97$, $P < 0.001$, time df = 5, $F = 2023.51$, $P < 0.001$, time*cultivar df = 10, $F = 0.035$, $P = 0.176$; *M. brassicae*: cultivar df = 2, $F = 17.30$, $P < 0.001$, time df = 8, $F = 2527.22$, $P<0.001$, time*cultivar df=16, $F=3.19$, $P=0.009$). With similar rank order as oviposition preference and abundance in the field, herbivores performed better on Badger Shipper plants (Figure 6).

Discussion

Our field data clearly show that intraspecific variation in secondary metabolite profiles of plants affects the abundance, richness and composition of the insect herbivore community. Rather than the total concentration of glucosinolates in cultivated plants, high concentrations of the C3 compound glucoiberin (3-methylsulfinylpropylglucosinolate) negatively

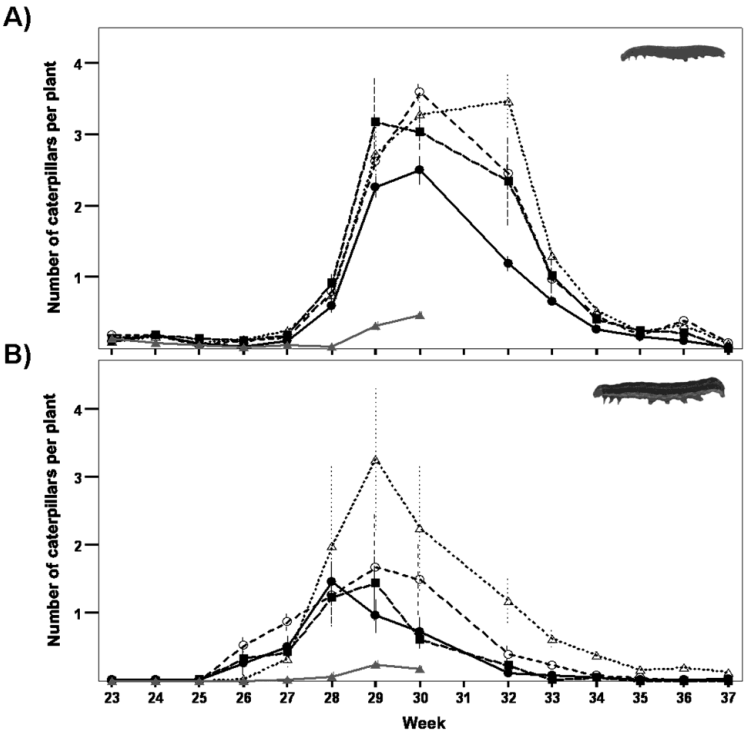

Figure 5. Population development of (A) *P. rapae* and (B) *M. brassicae* on *B. nigra* (grey line, closed triangle) and four cultivars of *B. oleracea* (black lines): Badger Shipper (open triangle), Christmas Drumhead (open circle), Lennox (closed square), Rivera (closed circle)) in a common garden experiment. Error bars are SE on plot level.

correlated with herbivore abundance, species richness and herbivore biodiversity. When glucosinolate profiles were dominated by the C4 compound glucoraphanin (4-methylsulfinylbutylglucosinolate), which is only one elongation step removed from the C3 compounds, plants harboured higher herbivore biodiversity. Specialists and generalists were not differentially affected by glucosinolate profiles. The underlying mechanisms are clear from the laboratory experiments. Both the specialist and the generalist Lepidopteran herbivore preferred to oviposit on plants with lower C3 glucosinolate concentrations and larvae had a reduced performance on plants with high concentrations of these compounds.

Herbivore community composition mediated by foliar glucosinolate profile

The effect of plant genotype on the community composition of higher trophic levels has been identified in an increasing number of plant species (Maddox and Root 1987; Fritz and Price 1988; Moyes et al. 2000; Johnson and Agrawal 2005, 2007; Bukovinszky et al. 2008). Our results support the notion that cultivars of plants not only differ in total herbivore abundance and species richness, but also account for differences in plant-associated biodiversity. Although examples exist for which environmental impact is equally strong or even more profound than genetic variation (Maddox and Root 1987; Fritz and Price 1988), genotypic variation in plants may be more influential than environmental variation in affecting arthropod communities (Johnson and Agrawal 2005; Bangert et al. 2006b). Heritable plant traits are thus a potent factor in shaping ecosystem biodiversity (Wimp et al. 2005; Witham et al. 2006). Plant size, architecture and phenological traits have all been shown to affect insect community composition and may even be more important than defence traits, as was e.g. shown for evening primrose *Oenothera biennis* (Johnson and Agrawal 2005). In 2007 we conducted a field study using the same cultivars as presented in this study, but then we destructively sampled the plants to assess whether plant biomass or number of leaves explained differences in herbivore abundance between the cultivars

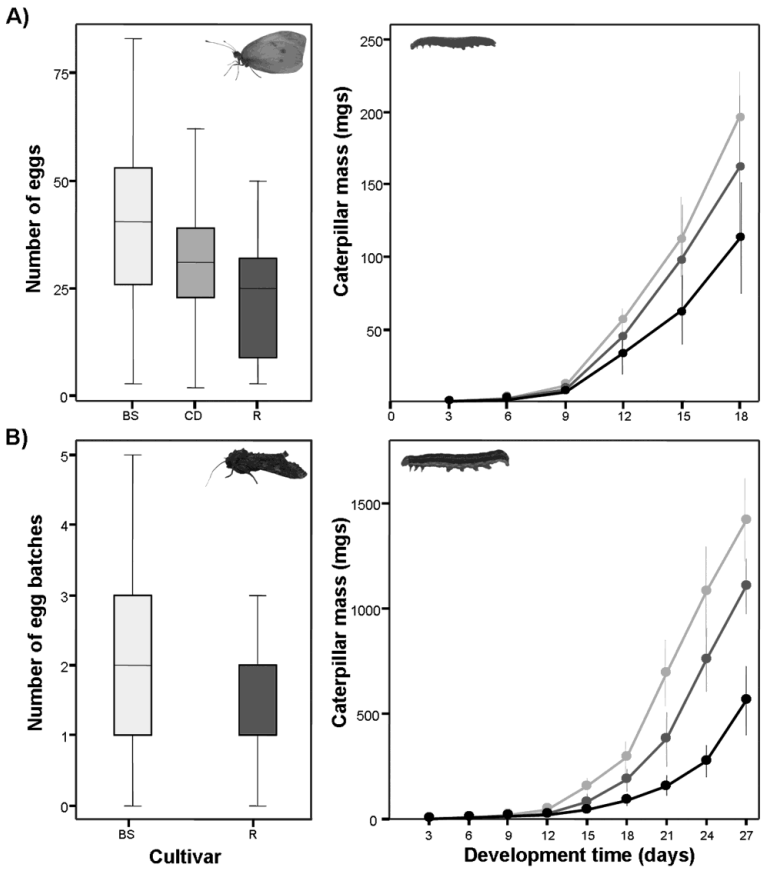

Figure 6. Oviposition preference for cultivars (left) and caterpillar performance (right) for the specialist butterfly *P. rapae* (A) and the generalist moth *M. brassicae* (B). Both species show similar preference and performance differences on *B. oleracea* cultivars, Badger Shipper (BS, light grey), Christmas Drumhead (CD, dark grey), Rivera (R, black).

(Poelman et al. unpublished data). Although cultivars differed in number of leaves and fresh weight, neither parameter correlated with herbivore abundance. Therefore, these morphological traits are not likely to explain the diversity parameters presented here and, thus, phytochemical composition of cultivars is more important than morphological traits in affecting the composition of the associated herbivore community. Only few studies have identified chemical compounds that mediate herbivore diversity. In Eucalyptus trees it was shown that high terpenoid concentration in leaves negatively correlated with arthropod biodiversity (Dungey et al. 2000). Gall aphid density, predator abundance and consequently total community biodiversity was negatively correlated with high leaf tannin concentration in cottonwood, *Populus* spp. (Bailey et al. 2006; Bangert et al. 2006a). In these studies only total concentration of a group of chemicals has been considered to be responsible for effects on biodiversity. However, plants may maintain a diversity of phytochemicals to confer a greater chance of resistance against all possible attackers (Jones and Firn 1991). In an evolutionary arms race with multiple attackers that adapt to specific chemical compounds, plants are under strong selection to produce novel compounds and maintain a diversity of compounds in their phytochemical profile. Specific compounds in the phytochemical profile may be active against specific herbivore species, thereby shaping the herbivore community on the plant.

Using our model system of cultivars that widely differed in glucosinolate profile but not total concentration of glucosinolates, we found that high concentrations of the C3 com-

pound glucoiberin in leaves of *Brassica oleracea* negatively affected herbivore abundance, richness and herbivore community composition. A higher concentration of the C3 compound glucoiberin correlated with a lower concentration of the C4 compound glucoraphanin as identified by the first principal component (PC) in the PCA analysis. Field et al. (2004) found that the ratio of C3 and C4 glucosinolates is genetically determined. By overexpressing genes encoding methylthioalkylmalate synthase (MAM) in *Arabidopsis*, concentrations of C3 glucosinolates decreased without changes in total glucosinolate concentration. The side chain of the C3 glucosinolates was elongated and the compound therefore biosynthetically linked with C4 compounds. Chemical diversity may not only have evolved by selection through a suite of attackers including different types of herbivores, but will also feed back to the behaviour of the herbivores themselves with consequences for herbivore biodiversity associated with the plants. Natural geographic variation within brassicaceous species consists of large variation in total glucosinolate concentration but includes large differences in glucosinolate profiles that differ in ratios of compounds with different side chain length (Kliebenstein et al. 2002; Gols et al. 2008a, b). Our results show that these ratios feed back differentially to the herbivore community and this implies that natural selection on these ratios is different over the geographic range (Mithen et al. 1995). One possible reason why this chemical variation is maintained, is that herbivore species show differential responses to the different concentrations of particular compounds.

Covarying effect for herbivores

Giamoustaris and Mithen (1995) and Van Leur et al. (2008) found that short side chain glucosinolates, such as the C3 compound glucoiberin, negatively affected host plant selection by generalist herbivores. At the same time some specialist herbivores preferred plants with high concentrations of short side chain glucosinolates. Herbivores with a similar degree of host plant specialization were found to covary in their responses to glucosinolates (Giamoustaris and Mithen 1995). In a meta-analysis on covariation among herbivore responses Leimu and Koricheva (2006) showed that generalist organisms covaried among themselves as did specialists. These patterns were, however, strong in mammalian herbivores and pathogens, and less profound for arthropods. Although this provides little support for host specificity of arthropods as a factor mediating differential responses to genetic variation in plants (Johnson and Agrawal 2007), studies on natural plant hybrids and their backcrosses support the hypothesis of differential response of herbivores based on insect host specificity (Whitham et al. 1999). Generalist herbivore species that are adapted to using a broad range of host types were found to be more common on hybrid plants that are intermediate to their parent host plants. Specialists were found to be less common on hybrids that resemble their specific host plant less (reviewed by Whitham et al. 1999), suggesting that high concentrations of host plant specific chemicals could lead to negatively correlated responses of generalists and specialists.

However, contrary to Giamoustaris and Mithen (1995) we found that generalist and specialist species covaried in the direction of response to the glucosinolate profiles. In our field study both specialists and generalists were less abundant on plants containing high concentrations of the C3-compound glucoiberin. Furthermore, *P. rapae* and *M. brassicae*, a specialist and generalist lepidopteran respectively, preferred to oviposit on plants with low glucoiberin concentration under laboratory conditions. Specialist and generalist herbivore performance also negatively correlated with glucoiberin concentration of laboratory plants. Although all herbivore species responded similarly with respect to abundance on cultivars, some species responded more strongly than others. The specialist cabbage aphid, *Brevicoryne brassicae*, responded stronger to cultivar differences than most of the other

species, but also avoided high concentrations of glucoiberin just like the other herbivores did. The low total glucosinolate concentrations that resulted from directional selection against these bitter tasting compounds by humans is likely to have reduced the concentration of glucosinolates in our cultivar model system below a threshold for natural discriminatory behaviour of herbivores. Nevertheless, our data clearly show that a single compound corresponds with resistance against the whole suite of herbivores and thereby affected herbivore biodiversity. Single compounds rather than chemical diversity of the plant were responsible for biodiversity effects. Plants may be selected to maintain the chemical diversity to increase the likeliness of possessing a compound that is active against many of its attackers. However, ratio differences in glucosinolates over a natural geographic range of *Brassica oleracea* did not correlate with herbivore community composition (Moyes et al. 2000). These in combination with our observations shown here suggest that the geographic variation in chemical diversity is likely maintained by other selection imposed by non-insect attack. Vertebrate and pathogen species attacking the plant may be a stronger selective force for maintenance of the chemical diversity observed in the field. Nevertheless, the resulting chemical profile feeds back on herbivore community diversity and our results show that the presence of particular compounds more strongly affects the plant-associated herbivore community than total concentration of glucosinolates.

Conclusion

Our results show that intraspecific variation in foliar glucosinolate profiles not only feed back on herbivore abundance and species richness, but may thereby affect herbivore community composition and biodiversity. More importantly, specific compounds that are biosynthetically linked correspond with these observations. The degree of host plant specificity of herbivores did not affect the direction of response of herbivores to variation in plant defence in our model system of cultivars with relatively low total glucosinolate concentration. Especially in agroecosystems glucosinolate profiles of brassicaceous plants therefore play a major role in structuring the diversity of the associated herbivore community. Biodiversity of herbivores has been identified to strongly affect higher trophic level biodiversity and thereby monocultures in agro-ecosystems that cover a major part of our globe profoundly structure the entire biodiversity on landscape level. With identifying a role for specific compounds in a phytochemistry profile of *Brassica* cultivars, it becomes a challenge to further elucidate the role of the C3 and C4 compound ratio in natural variation of brassicaceous plants. The knowledge of the regulation of compound elongation by a specific gene family (MAM like genes) in the *Brassica* related species *Arabidopsis thaliana* will in near future allow manipulation of compound ratios in wild type brassicaceous plants, with maintenance of the natural, high concentrations of glucosinolates and provide a tool to further elucidate a role for specific chemical compounds in nature.

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Appendix A. Pearson's correlation matrix for covariation among the abundance of herbivore species on field plots of *Brassica oleracea* cultivars. Boldface type present significant effects at $P < 0.05$.

		PR	PB	PX	MB	AG	MP	BB	PA	PU
Pieris rapae (PR)	*r*	1	-0.01	**0.50**	0.14	0.18	0.34	**0.36**	0.30	**0.42**
	P		0.98	**0.00**	0.44	0.31	0.06	**0.04**	0.10	**0.02**
Pieris brassicae (PB)	*r*		1	**0.37**	0.03	0.15	0.06	-0.01	**0.42**	0.19
	P			**0.04**	0.88	0.42	0.74	0.97	**0.02**	0.30
Plutella xylostella (PX)	*r*			1	0.21	0.30	0.31	0.23	**0.77**	**0.76**
	P				0.24	0.10	0.09	0.20	**0.00**	**0.00**
Mamestra brassicae (MB)	*r*				1	0.26	0.09	0.06	**0.36**	**0.44**
	P					0.15	0.61	0.75	**0.04**	**0.01**
Autographa gamma (AG)	*r*					1	**0.70**	**0.63**	0.24	0.27
	P						**0.00**	**0.00**	0.18	0.14
Myzus persicae (MP)	*r*						1	**0.56**	0.16	0.23
	P							**0.00**	0.38	0.21
Brevicoryne brassicae (BB)	*r*							1	0.32	0.32
	P								0.08	0.07
Phyllotreta atra (PA)	*r*								1	**0.90**
	P									**0.00**
Phyllotreta undulata (PU)	*r*									1
	P									

Chapter 4

Picture: Erik Poelman

Laboratory assays on differential attraction of *Cotesia* parasitoids by cultivars of *Brassica oleracea* reliably predict relative parasitism rates in the field

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Submitted

Abstract

Herbivore-induced plant volatiles (HIPVs) play an important role in host location of parasitoid wasps and may benefit the plant by top-down control of its herbivorous attackers. Although many studies have shown that accessions of plants differ in attractiveness to parasitoid wasps under controlled laboratory studies, few studies have confirmed that the most attractive accessions also sustain highest parasitism rates in the field. Here we tested whether in-flight preference of parasitoids for HIPVs from cultivars of *Brassica oleracea* in the laboratory reliably predicts the relative level of parasitism rates of herbivores feeding on these cultivars in the field.
In wind tunnel tests in the laboratory we ranked cultivars of *B. oleracea* for the preference of two congeneric parasitoids (*Cotesia glomerata* and *C. rubecula*) for their HIPVs. The cultivars were then compared for their relative parasitism rates of caterpillars in the field. Throughout the growth season in the field we infested the different cultivars with *Pieris* caterpillars on a weekly basis. The caterpillars were recollected after three days, dissected and scored for the rate of parasitism.
Parasitism pressure on herbivores was highest during weeks when natural *Pieris* populations consisted of early instar caterpillars. Cultivars of *B. oleracea* that we identified as most attractive in the laboratory also sustained highest proportions of parasitism in the field. Headspace analysis of the *B. oleracea* cultivars revealed that upon herbivory only the relative levels of methyl salicylate and methyl thiocyanate corresponded with the in-flight preference of the wasps. Cultivars that were more attractive to wasps emitted higher concentrations of these two compounds in response to *P. rapae* herbivory. Our data show that the relative degree of parasitoid attraction to HIPVs of *B. oleracea* cultivars, as assessed under controlled laboratory conditions, corresponds with parasitism rates in the field.

Key words: indirect defence, volatiles, methyl salicylate, methyl thiocyanate, *Cotesia glomerata*, *Cotesia rubecula*

Introduction

Since the first report appeared only two decades ago (Dicke and Sabelis 1988), indirect plant defences have become a central issue in the study of plant-insect interactions (D'Allesandro and Turlings 2006; Heil 2008). In response to herbivore attack, plants emit volatiles that attract natural enemies of the herbivore to the plant. The exploitation of volatile information by natural enemies leads to a top-down control of herbivorous plant attackers and thereby may benefit plants that emit detectable and reliable volatile information (Vet and Dicke 1992; Dicke and Vet 1999). In a wide range of plant species it has now been identified that herbivore-induced volatile emission considerably differs in qualitative and quantitative aspects from volatiles emitted by undamaged plants and results in increased attraction of parasitoids or other natural enemies of herbivores (Takabayashi and Dicke 1996; Heil 2008). Detailed studies of molecular mechanisms and biosynthetic pathways has resulted in the identification of a number of chemical compounds that may function as a plant-produced cue indicating herbivore presence (Ibrahim et al. 2005; Kappers et al. 2005; Snoeren et al. 2007; Mumm et al. 2008). It is recognized that volatile information may provide wasps with information on the identity of the herbivore, such as species (Blaakmeer et al. 1994; Geervliet et al. 1996; De Moraes et al. 1998; de Boer et al. 2004), instar (Takabayashi et al. 1995) or on its parasitization status (Fatouros et al. 2005). Herbivore-induced plant volatiles (HIPVs) can be highly specific and reliable cues for parasitoids or predators during host location. There is empirical support that the emission of HIPVs can result in enhanced resistance of plants (Thaler 1999; Halitschke et al. 2008; but see Karban 2007) and may result in higher plant fitness (van Loon et al. 2000; Smallegange et al. 2008).

However, studies on indirect plant defence have thus far relied heavily on controlled laboratory setups. One of the major concerns of relying on laboratory results is whether they provide a reliable measure of parasitoid responses to volatiles in the field. Setups such as wind tunnels or Y-tube olfactometers offer wasps two-choice situations with highly standardized conditions. However, the array of abiotic and biotic factors in agricultural and natural fields may hamper the effectiveness of volatile information (Takabayashi et al. 1994; Hunter 2002). The behavioural response of parasitic wasps to volatile emission by plants has been found to strongly vary with fluctuating abiotic conditions such as barometric pressure, humidity, light conditions etc. (Steinberg et al. 1992; Gouinguene and Turlings 2002; Takabayashi et al. 1994). More importantly, biotic agents that may induce the plant to produce different blends of volatiles vary dramatically in time, abundance and severity of the damage they impose. Similarly, chemicals from neighbouring plants may conceal HIPVs to parasitoids (Hunter 2002; Dicke et al. 2003; de Boer et al. 2008). Laboratory studies that incorporate some level of natural complexity in their tests, such as mixing different plant species (Gols et al. 2005; Bukovinszky et al. 2007), or inducing plants with a mix of herbivore species (Shiojiri et al. 2001; Rodriguez-Saona et al. 2005; Moayeri et al. 2007; Rasmann and Turlings 2007; de Boer et al. 2008), demonstrated that increased chemical complexity may result in less distinct preferences by parasitoids.

Surprisingly few studies have investigated whether HIPVs lead to a higher degree of parasitization or predation of herbivores in the field. The majority of these field studies aimed at supporting a prime assumption of indirect defence by identifying that volatile compounds indeed attract parasitoids or predators in the field. By using traps baited with chemical compounds (James and Price 2004; James and Grasswitz 2005) or herbivores and chemicals as elicitors of volatile responses of plants (De Moraes et al. 1998; Thaler 1999; Bernasconi-Ockroy et al. 2001; Kost and Heil 2008) an enhanced attraction of parasitoids

to the treated plants has been found. However, there is limited information on whether plant cultivars or genotypes identified as highly attractive to parasitoids in laboratory set-ups or field assays also sustain a higher degree of parasitization of herbivores in the field (but see Bradburne and Mithen (2000) for a field study on aphid parasitoids; Rasmann et al. (2005) for a field study on entomopathogenic nematodes and Halitschke et al. (2008) for a study on predators).

Here we tested whether wind tunnel preference tests under controlled conditions may be reliable predictors of parasitoid responses to intraspecific variation in plant volatiles under field conditions. We focused on the intensively studied tri-trophic interaction between *Brassica* plants, *Pieris* butterflies and their parasitoid wasps *Cotesia glomerata* and *Cotesia rubecula* (Steinberg et al. 1992; Brodeur et al. 1996; Geervliet et al. 1996, 1997; Mattiacci et al. 2001; Smid et al. 2002). *Cotesia glomerata* is a gregarious endoparasitoid of caterpillars of several *Pieris* species, but prefers *P. brassicae* as its host, and lays clutches of 5-35 eggs in a single caterpillar. The congeneric wasp *Cotesia rubecula* is a specialist on *Pieris rapae* caterpillars that live solitarily on host plants and this wasp lays only a single egg per host (Brodeur et al. 1996). Both parasitoid species are known to strongly discriminate between plant species that are infested with their herbivorous host (Geervliet et al. 1996). Variation in HIPVs between cultivars is known to elicit discriminatory behaviour of wasps, but results of wind tunnel tests are often showing a less profound discriminatory response compared with wasps that are tested for their response to volatile emissions from different plant species (Geervliet et al. 1997). Intraspecific variation in volatile emission may be more subtle than variation between plant species (Takabayashi and Dicke 1996). Moreover, these subtle differences may be more easily overruled/ disturbed by biotic and abiotic factors affecting HIPVs emissions in the field.

We tested the two congeneric parasitoid species for their in-flight preference for HIPVs of cultivars of *B. oleracea* in two-choice wind tunnel assays. From these assays we constructed a ranking of attractiveness and tested whether caterpillars on high-ranking cultivars also had a higher risk of parasitization in the field. During two field seasons we experimentally studied the frequency of parasitism on the cultivars. In addition, we sampled the dynamic headspace of laboratory-reared plants infested with caterpillars. These data were analyzed using a multivariate approach, which provided a comprehensive view on constitutive and induced differences in volatile profiles between the cultivars (van Dam and Poppy 2008). We specifically addressed the following questions: 1) Are parasitoids consistently attracted to particular cultivars of *B. oleracea* under both laboratory and field conditions? 2) Does a natural population of parasitoids effectively parasitize naturally fluctuating host populations? 3) If preference for certain cultivars is observed, can this be attributed to differences in volatile emission by these cultivars? 4) Are attractive cultivars sustaining better wasp performance than less attractive cultivars?

Material and Methods

Insects and plants

In laboratory and field experiments we used the following white cabbage cultivars (*Brassica oleracea* var. *alba* L.; donors are given in brackets): Badger Shipper, Christmas Drumhead (Centre for Genetic Resources, CGN-Wageningen, The Netherlands), Lennox and Rivera (Bejo Zaden BV, Warmenhuizen, The Netherlands). Seeds for the common garden experiment were directly sown into peat soil cubes and placed in a greenhouse (L16:D8 photoperiod with SON-T light, 500 μmol $m^{-2}s^{-1}$ in addition to daylight, at 18–26 ºC and 40-70% relative humidity). When seedlings were three weeks old, the trays containing the plants were placed outside during the day to acclimatize the plants to field condi-

tions. The plants were transplanted with their soil cubes into the soil at the experimental site when they were five weeks old.

Seeds of plants used in laboratory experiments were germinated on peat soil (Lentse potgrond, No. 4) and transferred as two-week-old seedlings to 1.45 L pots containing the same potting soil. Pots were placed in a greenhouse (L16:D8 photoperiod with SON-T light (500 µmol $m^{-2}s^{-1}$ in addition to daylight), at 18–26 ºC and 40-70% relative humidity). When the plants were four weeks old, they were fertilized each week by applying 100 ml nutrient solution to the soil (KristalonTM®, concentration 3 g/l, 16N: 6P: 20K: 3Mg). We used six-week-old plants that had ten true leaves in parasitoid preference and performance experiments.

For wind tunnel preference experiments on parasitoid wasps, the plants were infested with ten second instar caterpillars of *Pieris rapae* L. (Pieridae) that were allowed to feed on the plants for 24 hours prior to the experiment.

The caterpillars and parasitoids originated from the respective cultures at the Laboratory of Entomology, Wageningen University. They are maintained on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* L. cultivar Cyrus) in a climatized room at 20-22 ºC, 50-70% relative humidity and a L16:D8 photoperiod with SON-T light (500 µmol $m^{-2}s^{-1}$ in addition to daylight). For the rearing of *Cotesia rubecula* (Hymenoptera: Braconidae), *P. rapae* is used as a host and *C. glomerata* is reared on caterpillars of *P. brassicae*.

Laboratory studies

Wind tunnel assay

Preference tests with the two congeneric parasitoids for cultivars of *Brassica oleracea* were carried out in a wind tunnel as previously described by Geervliet et al. (1994). Wasps were studied in six dual-choice situations in which single plants of two cultivars were placed in pairs upwind in the tunnel. We tested in-flight preference of the wasps for volatiles from undamaged plants against *P. rapae*-damaged plants for three *B. oleracea* cultivars separately (Christmas Drumhead, Rivera, Badger Shipper) as well as in dual-choice tests between *P. rapae*-induced plants of the three cultivars (3 combinations). We tested 8 to 10 naive wasps per set of plants and the position of the plants was swapped after 4 or 5 consecutive tests. Each wasp was transferred from the rearing cage to the release cylinder in the wind tunnel on a Brussels sprouts leaf piece. The release cylinder with open ends was positioned at approximately 70 cm downwind of the test plants. From the time of release in the open glass cylinder, wasps were allowed 5 minutes to take off, fly out of the cylinder and land on a test plant upwind, which was considered as a choice for one of the treatments. Wasps that did not respond within 5 minutes were considered as 'not responding'. Each of the six treatment combinations was tested with at least six sets of plants on six different days. Wind speed in the tunnel was set at 0.1 m/s, light conditions varied from 24 to 26 µmol $m^{-2}s^{-1}$, and ambient temperature varied between 21 and 27 °C. Relative humidity in the wind tunnel was kept above 50%.

Performance test

Performance of the two parasitoid species in their host *P. rapae* was compared when hosts were feeding on one of three cultivars of *B. oleracea* (Christmas Drumhead, Rivera, Badger Shipper). At the start of the experiment we offered second instar *P. rapae* caterpillars to female wasps for parasitization. When a wasp was observed stinging a caterpillar, the caterpillar was collected. Each female wasp was allowed to sting up to a maximum of ten caterpillars. As soon as we had collected 150 parasitized caterpillars, we randomly dis-

tributed them over three cages (50x50x60 cm) so that each cage contained 50 caterpillars at the start of the experiment. Each of the cages contained eight plants of one of the cultivars. The parasitized caterpillars were allowed to move freely on these plants and new plants were added when over 70% of the leaf tissue was consumed to allow the caterpillars to feed *ad libitum* at all times. We checked the cages for emerging parasitoid larvae every day. When wasp larvae had emerged from their caterpillar host, the number of days since the start of the experiment was recorded and the cocoons were collected. The cocoons were placed in 2.2 ml Eppendorf tubes that were closed with a piece of cotton wool and stored in a climate cabinet (22±1 °C). The cocoons were checked every day for emerging wasps. When a wasp had emerged from its cocoon the date was recorded and the wasp was stored at -20 °C. For each of the wasps we measured dry mass, determined their sex and for *C. glomerata* we also scored the clutch size by counting the number of cocoons per caterpillar. For both wasp species the performance experiment was replicated two months after the first experiment. Data of the two experiments were combined, because there were no significant differences between replicates regarding the number of days until larvae emerged (Mann Whitney U test: *C. glomerata*: Z = -1.16, P = 0.25; *C. rubecula*: Z = -0.63, P = 0.53).

Field studies

Study site

In 2005 and 2006 we established a field experiment in an agricultural field in the vicinity of Wageningen, The Netherlands. Forty plots (6 x 6 m), each planted with a monoculture of one of the four cultivars in 2005 (Christmas Drumhead, Rivera, Badger Shipper, Lennox) or one of two cultivars in 2006 (Rivera and Badger Shipper) were established using a randomized design. Five-week-old seedlings rooted in soil cubes were transplanted to the field in week 19 in both years. We planted 49 plants in a square of 7 x 7 plants per plot with 75 cm of bare soil between plants. Plots were isolated from each other by a strip of 6 m that was sown with a grass mixture of *Lolium* and *Poa* species. The central nine plants of each plot were surveyed weekly for the presence of naturally colonizing herbivores (Poelman et al. 2008a, b). Here we only present the abundance of *P. rapae* and *P. brassi-*

Table 1. Larval performance parameters (mean (SE)) of *Cotesia glomerata* and *C. rubecula* in *Pieris rapae* hosts that fed on three cultivars of *Brassica oleracea*. Bold face type indicate significant terms in Kruskal-Wallis tests ($\alpha = 0.05$).

	Christmas Drumhead	Rivera	Badger Shipper	χ^2	P
Cotesia glomerata					
Nr. of cocoon clutches found	58	62	54		
Clutch size	17.31 (1.05)	17.87 (0.82)	18.40 (0.95)	1.04	0.59
Nr. of days until larva emerged from the caterpillar	13.91 (0.15)	15.34 (0.14)	15.07 (0.12)	45.72	**< 0.001**
Nr. of days until wasp emerged from the cocoon	20.06 (0.14)	21.73 (0.12)	20.98 (0.11)	60.37	**< 0.001**
Female to male ratio within a clutch	0.56 (0.04)	0.51 (0.05)	0.46 (0.04)	3.66	0.16
Dry mass	0.47 (0.02)	0.48 (0.01)	0.50 (0.02)	3.31	0.19
Cotesia rubecula					
Nr. of cocoons found	60	61	57		
Nr. of days until larva emerged from the caterpillar	10.01 (0.09)	11.19 (0.10)	10.3 (0.08)	70.86	**< 0.001**
Nr. of days until wasp emerged from the cocoon	16.42 (0.09)	18.17 (0.11)	16.6 (0.09)	87.49	**< 0.001**
Dry mass	0.80 (0.01)	0.93 (0.13)	0.79 (0.01)	0.73	0.70

cae in the field over the study period during which we carried out the parasitism experiment as described in the next paragraph. We linked natural population dynamics of these herbivorous hosts of *Cotesia* parasitoids to the rate of parasitism identified in the parasitism experiment to show the relationship between natural herbivore abundance and parasitoid pressure on these herbivores.

Parasitism experiment

In the years 2005 and 2006 we experimentally measured the rates of parasitism of *Pieris* caterpillars feeding on different cultivars of *B. oleracea* from week 24 until week 36. Each week a single plant per field plot was infested with ten second instar *Pieris* caterpillars that were placed on the same leaf. Every week we used a different plant in the field plot, excluding the central nine plants that were used for monitoring of natural herbivore abundance. In week 24 we started with *P. brassicae* and alternated this host with *P. rapae* every other week. In our field experiments we included *P. brassicae* in addition to *P. rapae* since it is the preferred host of *C. glomerata.* The use of both *Pieris* species provided the opportunity to test whether differences in parasitization rates of caterpillars on cultivars was affected by herbivore host preference of *C. glomerata*. The caterpillars were recollected four days after they were released in the field. To avoid collection of naturally occuring *Pieris* caterpillars, we only collected caterpillars that were in second or early third instar stage. We dissected the caterpillars under a stereomicroscope, recorded whether caterpillars were parasitized by *C. glomerata* or *C. rubecula* and how many parasitoid eggs or larvae a caterpillar contained. Parasitization of caterpillars by either wasp species was discriminated by egg size and mandible size of the parasitoid larvae. Eggs of *C. rubecula* are about twice as large as those of *C. glomerata* and its 1st instar larvae have conspicuous mandibles (Laing and Corrigan 1987).

Volatile analysis

To identify candidate chemical compounds that may have explained differences in the attraction of parasitoid wasps to different cultivars of *B. oleracea*, we collected volatiles from control and *P. rapae*-induced plants (induced by 24 hours of feeding) of the cultivars Christmas Drumhead, Rivera, and Badger Shipper. The six-week-old plants were placed in

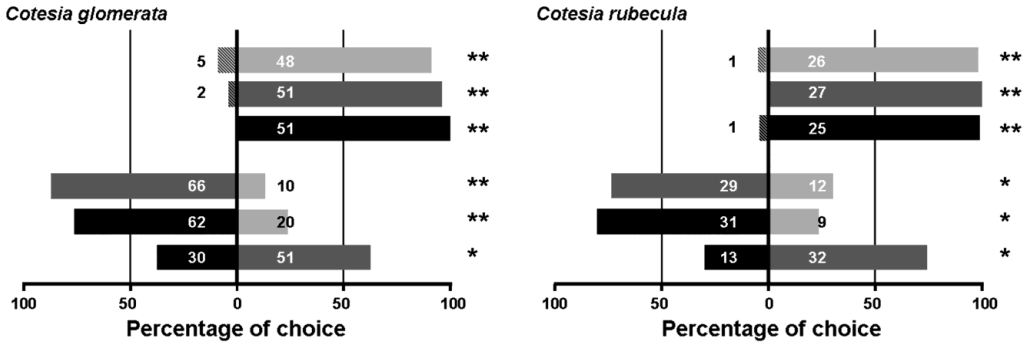


Figure 1. Preference of *Cotesia glomerata* (left) and *Cotesia rubecula* (right) for cultivars of *Brassica oleracea* as tested in dual-choice wind tunnel tests. Numbers in the bars indicate the number of wasps that made a choice for that specific treatment. The top three sets of bars present a choice test between an uninfested (left) and a *Pieris rapae*-infested plant (right) in separate tests for Badger Shipper (light grey), Christmas Drumhead (dark grey) and Rivera (black). The lower three sets of bars present separate choice tests between *P. rapae*-infested plants of two different cultivars. * $P < 0.05$, ** $P < 0.001$.

a 17 L bell-shaped glass jar in a climate-controlled cabinet (21±1 °C, 60-70% relative humidity). The jars were supplied with a constant flow of 300 ml of pressurized air (Hoekloos, The Netherlands) that was cleaned over a Zero Air generator (Parker Hannifin Corp, Tewksbury, MA, USA). Plant volatiles were trapped on a steel trap filled with 150 mg of Tenax TA and 150 mg Carbopack B using a vacuum pump with a flow rate of 100 ml/min. The traps were removed after 30 min and capped until analysis. Each of the six plant treatments was replicated nine times. On a single day we collected volatiles in seven series, each with four plants. Volatiles were also trapped from air that had passed through an empty glass chamber to determine background levels of volatiles.

The steel traps were placed in an automated thermodesorption unit (model Unity, Markes, Pontyclun, UK) that desorbed the volatiles from the traps at 200 °C during 10 min. The desorbed volatiles were focused on a cold trap (-10°C). After 1 min of dry purging, the cold trap was heated for 3 min (270 °C) and the volatiles introduced into a GC-MS (model Trace, ThermoFinnigan, Austin, Texas, USA). The GC was set to a 1:4 split rate and we used a 30 m x 0.32 mm ID RTX-5 Silms column with a film thickness of 0.33 µm. The temperature program used was: 40 °C to 95 °C at 3 °C /min, then to 165 °C at 2 °C / min and finally to 250°C at 15 °C /min. The volatile compounds were detected by the MS operating at 70 eV in EI mode. Compound mass spectra were acquired in a full scan mode (33-300 AMU, 3 scans/s). The compounds were identified by their mass spectra using deconvolution software (AMDIS) in combination with Nist 98 and Wiley 7th edition spectral libraries, and by comparing their linear retention indices. The mass spectra and linear retention indices were compared with values reported in literature, obtained by interpolating homologous series, or by analyzing of reference compounds (farnesene, benzonitrile, dimethyldisulfide, dimethyltrisulfide and limonene; Sigma-Aldrich, Zwijndrecht, NL). The peaks of identified compounds in the chromatogram were integrated by Xcalibur software (version 1.3, Finnigan). To exclude potential interference by co-eluting compounds, specific quantifier ions were selected for each individual compound of interest. In general, these quantifier ions were similar to the most intense model ions extracted from the raw mass spectrum by AMDIS. We used the integrated absolute signal of the quantifier ion for comparison between the treatments. Peak areas in each sample were divided by the total volume of air (ml) that was passing over the traps and divided by the aboveground fresh plant biomass, which was assessed after volatile trapping. In this way we corrected for differences in air flow over the traps and size of individual plants.

Statistical analysis

We used Generalized Linear Models (GLM) in the statistical programme GenStat 8th Edition to analyze whether experimentally introduced *Pieris* caterpillars in the field differed in parasitization rate between cultivars. Since the setup of the field experiments was different for each of the two years, we used separate models for each year. First we tested whether the number of caterpillars recollected from the field was different for the cultivars tested. The number of recollected caterpillars was included in the model as a binomially distributed dependent variable with fixed binomial totals of 10, the number of caterpillars used to infest a single plant. Second, to test for differences in parasitism rates of these recollected caterpillars, we constructed a new model. Here we included the number of parasitized caterpillars as a binomially distributed dependent variable with the fixed totals of the number of recollected caterpillars on that field plot. We present full models in each of these analyses including the factors: cultivar (Christmas Drumhead, Rivera, Badger Shipper, Lennox), study week (24-36), caterpillar species (*P. rapae*, *P. brassicae*) and their interactions in a full factorial design. However, the interaction terms between "caterpillar" and "week"

were excluded since caterpillar species (*P. rapae* or *P. brassicae*) were infested on plants in different weeks.

In the analysis of headspace composition of the cultivars, we were interested in which compounds corresponded to the preference of parasitoids found in wind tunnel and field experiments. To function as a reliable cue for parasitoids, we considered that these compounds should be (1) consistently present in all control or *P. rapae* induced samples of a cultivar, (2) induced upon *P. rapae* herbivory in the same direction in all cultivars (increased or decreased). We therefore first identified which compounds were detected in each of the nine replicates of an induced or control treatment per cultivar. We removed all compounds that did not comply with this criterion from further analysis. Thereafter we performed PCA analysis on control plants of each of the cultivars, PCA analysis on the induced plants of the cultivars and PCA analysis on control and induced plants within a cultivar. The PCA analyses first identified whether headspace compositions of cultivars or treatments were distinctly different from each other. We then checked the loading scores of each of the compounds in a PCA analysis to identify which of the compounds contributed most to explaining the variation among samples. The compounds that were identified in each of these PCA analyses were listed (bold face type in Table 3) and then subjected to criterion (2). Compounds from this shortlist that increased or decreased consistently in concentration in the three cultivars after *P. rapae* feeding (2) were further tested for significant differences between treatments. We used ANOVA to test whether cultivars and induction treatment significantly affected the concentrations of the selected compounds. Post-hoc Tukey tests were performed for the factor cultivar.

Results

Laboratory studies

In total 78% of the tested wasps landed within 5 min on one of the plants upwind in the wind tunnel experiments. *Cotesia glomerata* and *C. rubecula* both clearly preferred plants infested with their host *P. rapae* over undamaged plants (Figure 1) (Christmas Drumhead: *C. glomerata*: $Z = 6.59$, $P < 0.001$, *C. rubecula*: $Z = 5.00$, $P < 0.001$; Rivera: *C. glomerata*: $Z = 7.00$, $P < 0.001$, *C. rubecula*: $Z = 4.51$, $P < 0.001$; Badger Shipper: *C. glomerata*: $Z = 5.77$, $P < 0.001$, *C. rubecula*: $Z = 4.62$, $P < 0.001$). When infested plants of the cultivars were compared among each other in two-choice tests, *C. glomerata* and *C. rubecula* also responded with a similar preference for cultivars. Christmas Drumhead was preferred over Rivera (*C. glomerata*: $Z = 2.22$, $P = 0.026$; *C. rubecula*: $Z = 2.68$, $P = 0.007$) and both cultivars were preferred over Badger Shipper (Christmas Drumhead over Badger Shipper: *C. glomerata*: $Z = 6.309$, $P < 0.001$; *C. rubecula*: $Z = 2.50$, $P = 0.012$; Rivera over Badger Shipper: *C. glomerata*: $Z = 4.53$, $P < 0.001$; *C. rubecula*: $Z = 3.32$, $P < 0.001$).

Larvae of *C. glomerata* as well as *C. rubecula* developing in *P. rapae* performed best on the preferred cultivar Christmas Drumhead. However, the second most attractive cultivar, Rivera (Figure 1), sustained poorest larval parasitoid performance as judged by the number of days until parasitoid larvae emerged from their host and the number of days until adult wasps emerged from their cocoons (Table 1).

Field studies

Parasitism rates as recorded in experimentally released caterpillars had two peaks over the study period in both study years (Figure 2). These patterns were remarkably similar for the two years and the first peak coincided with the increase in population size of naturally occurring *Pieris* caterpillars. The second peak of parasitism frequency occurred six weeks later (week 34). Within these natural fluctuations of parasitism pressure, cultivars differed

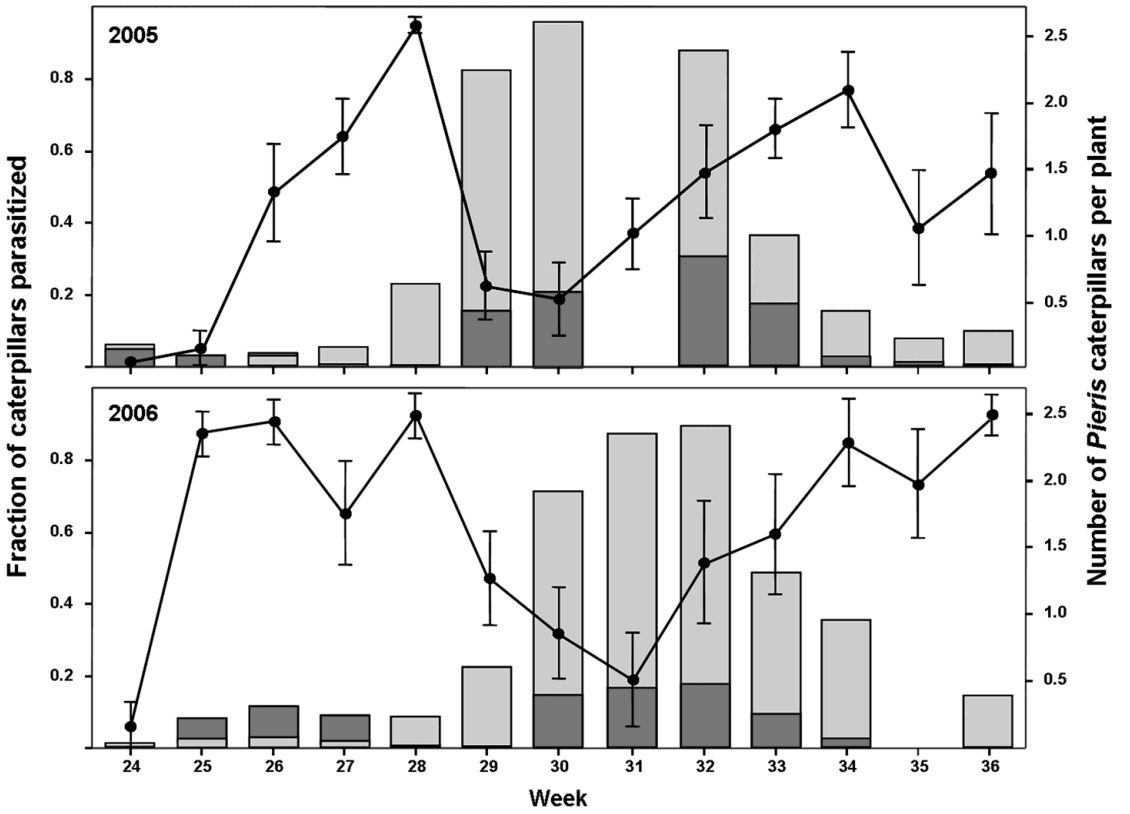


Figure 2. Fraction of parasitized *Pieris* caterpillars that had been experimentally introduced in field plots, as assessed over the different study weeks in the year 2005 (top) and 2006 (bottom), plotted together with the natural population dynamics of *Pieris* species in the field. The dots and drawn line represent the average (±SE) fraction (left y-axis) of experimentally introduced *Pieris* caterpillars that were parasitized in the respective experimental week. The bars represent the natural population size in number of caterpillars per plant of *Pieris rapae* (light grey) and *P. brassicae* (dark grey), plotted on the right y-axis. Natural *Pieris* abundance in a field plot was monitored on different plants than the plants used for the parasitization experiment. In 2005 week 31 and in 2006 week 35, herbivore numbers were not monitored.

in the fraction of caterpillars that were parasitized. In 2005 cultivar Christmas Drumhead had significantly higher levels of parasitism than the other three cultivars (Figure 3, Table 2). In 2006 we tested the intermediately attractive cultivar Rivera against the least attractive Badger Shipper and found a significantly higher rate of parasitism on Rivera plants (Figure 3, Table 2). In the weeks during which we infested plants with *P. rapae* caterpillars, the fraction of caterpillars parasitized was not different from those in which we infested plants with *P. brassicae* in 2005, but *P. brassicae* caterpillars were parasitized more frequently than *P. rapae* caterpillars in 2006. However, the differences in parasitism frequencies between cultivars were similar for both *Pieris* species. The number of caterpillars recollected differed between weeks and we recollected more caterpillars on Badger Shipper and Christmas Drumhead than on Rivera or Lennox plants in 2005 and 2006. Proportions of caterpillars recollected were higher for *P. brassicae* than for *P. rapae* in both study years (Table 2).

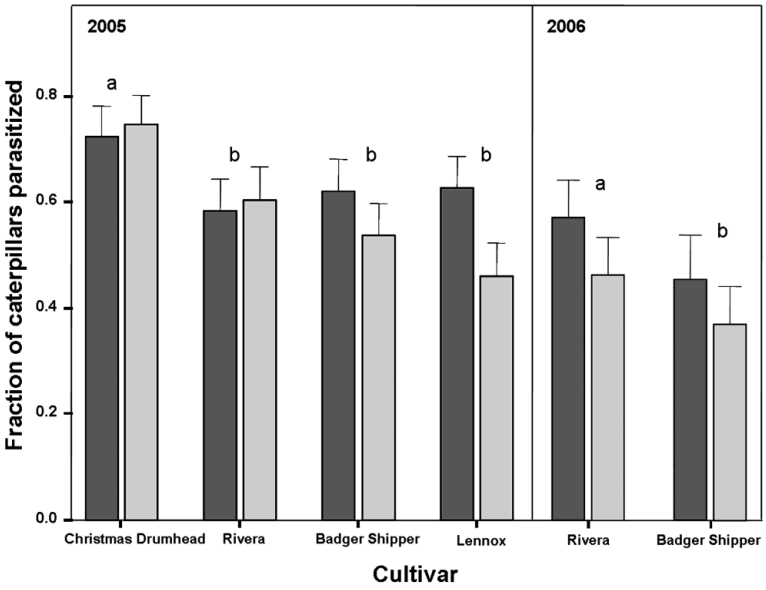


Figure 3. Parasitization rates of experimentally introduced *Pieris* caterpillars on different *B. oleracea* cultivars in the field pooled for all 13 experimental weeks per study year, 2005 (left), 2006 (right). Bars represent the average (±SE) fraction of parasitized caterpillars for *P. brassicae* (dark grey) and *P. rapae* (light grey). Statistical tests (GLM) were performed separately for years, different letters above the bars present significant differences between parasitism fractions for cultivars within a year.

Volatiles

Using GC-MS we identified 440 compounds in the headspaces of control and *P. rapae*-induced plants of the *B. oleracea* cultivars Christmas Drumhead, Rivera and Badger Shipper. Only 43 of these compounds were present in all replicates of at least one of the six treatment groups and were included in further analyses (Table 3). Control plants of Rivera released a different blend of volatiles than control plants of Badger Shipper and Christmas Drumhead (Figure 4A). The differences between cultivars were most pronounced on the first Principal Component (PC) axis that explained 77% of the variation in the dataset. The separation between cultivars was more pronounced when plants had been subjected to 24 h of *P. rapae* feeding. The volatile profile of Rivera became distinctly different from the profile of the other two cultivars, whereas the profiles of Christmas Drumhead and Badger Shipper remained relatively similar to each other (Figure 4B). Here, the first PC axis explained 70% of the variation in the dataset and was also responsible for the best separation between the different cultivars. In the PCA analysis on control plants as well as the analysis on induced plants, the same compounds showed a high loading on the first PC-axis, which is presented in a single loading plot (Figure 4C). Eight (mono)terpenes and the ester 3-hexen-1-ol-acetate contributed most to the separation between cultivars (Figure 4C, Table 3). When comparing the composition of the volatile blend of plants induced by *P. rapae* feeding with undamaged plants of the same cultivar, we identified also a few other compounds that contribute most to separation between induction and control treatments. These compounds included methyl salicylate and methyl thiocyanate (Table 3). None of the terpenes that were identified by PCA analysis to correspond to differences between induced and control plants showed consistent changes after herbivory in the three cultivars. In Christmas Drumhead most of the terpenes were emitted in lower amounts after herbivory, whereas the same compounds were emitted in higher amounts in herbivory-induced plants of Rivera and Badger Shipper than in control plants. We established that only methyl salicylate and methyl thiocyanate emission levels were consistently increased upon herbivory in the three cultivars and that their emission rates differed between cultivars. More importantly, their emission rates also corresponded to the differences in attractiveness of the cultivars to parasitoids as observed in the laboratory study. The most attractive

Table 2. Generalized Linear Model deviance table for the fraction of caterpillars recollected and parasitized in the field season 2005 and 2006. The terms including interactions between caterpillar and week were excluded in the Full model since caterpillar species (*P. rapae* or *P. brassicae*) were infested on plants in different weeks. Bold face type indicate significant terms (α = 0.05).

	Full model		Factor						Interaction			
			Cultivar (1)		Caterpillar (2)		Week (3)		1 * 2		1 * 3	
2005			d.f = 3		d.f. = 1		d.f. = 12		d.f = 3		d.f. = 36	
	deviance		deviance	P	deviance	P	deviance	P	deviance	P	deviance	P
Fraction of recollected caterpillars	2009.79	d.f. = 415	19.79	< **0.001**	156.29	< **0.001**	333.31	< **0.001**	12.85	**0.005**	71.67	< **0.001**
Fraction of parasitized caterpillars	1944.22	d.f. = 383	43.73	< **0.001**	1.85	0.174	1008.53	< **0.001**	129.34	< **0.001**	9.30	**0.026**
2006			d.f. = 1		d.f. = 1		d.f. = 12		d.f. = 1		d.f. = 12	
	deviance		deviance	P	deviance	P	deviance	P	deviance	P	deviance	P
Fraction of recollected caterpillars	2537.99	d.f. = 519	8.73	**0.003**	343.42	< **0.001**	561.08	< **0.001**	1.40	0.24	29.05	**0.004**
Fraction of parasitized caterpillars	2631.53	d.f. = 485	51.96	< **0.001**	61.43	< **0.001**	1188.67	< **0.001**	74.18	< **0.001**	73.55	< **0.001**

cultivar, Christmas Drumhead, emitted the highest amounts of methyl salicylate and methyl thiocyanate. These amounts were significantly higher than in Badger Shipper plants, but not statistically different from Rivera plants (ANOVA, methyl salicylate: cultivar: d.f. = 2, F = 7.14, P = 0.002; induction: d.f. = 1, F = 33.37, P < 0.001, cultivar * induction: d.f. = 2, F = 6.56, P = 0.003; Methyl thiocyanate: cultivar: d.f. = 2, F = 6.62, P = 0.003, induction: d.f. = 1, F = 9.76, P = 0.003, cultivar * induction: d.f. = 2, F = 1.74, P = 0.186).

Discussion

Our results presented here demonstrate that the relative attractiveness of HIPVs of *B. oleracea* cultivars as identified in controlled laboratory tests parallels the differences in the degree of parasitization of herbivores in the field. The preference of *C. glomerata* and *C. rubecula* wasps for *P. rapae*-induced plants of the *B. oleracea* cultivar Christmas Drumhead corresponded with higher parasitism frequencies of *Pieris* caterpillars compared to other cultivars in the field. In weeks when the preferred host *P. brassicae* as well as in weeks when the sub-optimal host *P. rapae* of *C. glomerata* was used, caterpillars were parasitized more frequently on Christmas Drumhead compared to other cultivars. Despite the volatile noise that may have been caused by the natural insect community present on a plant, cultivars differed in the proportions of experimentally applied caterpillars that were parasitized. The second most attractive cultivar as identified in wind tunnel tests, i.e. Rivera (Figure 1), sustained only significantly higher frequencies of parasitism compared to Badger Shipper in the field test of 2006 when Christmas Drumhead plants were absent. Fractions of parasitized caterpillars were highest in those weeks when natural herbivore populations consisted mostly of caterpillars in the early instars. Attractive cultivars produced more methyl salicylate and methyl thiocyanate upon induction by *P. rapae* caterpillars. Although the most attractive cultivar also sustained highest performance of the parasitoids, their performance was poorest on the second most attractive cultivar, i.e. Rivera. The ranking orders of preference and performance do therefore not correspond completely.

In the field, parasitization rates strongly fluctuated, but were very similar between years. The fluctuations coincided with the dynamics of herbivore host populations such that when early instar caterpillars were present, parasitism pressure was at its maximum. Our data show that naturally occurring herbivore pests on agricultural fields were effectively parasitized by natural parasitoid populations. At the moment when natural herbivore attack on the agricultural field was at its maximum, the two *Cotesia* parasitoids parasitized over 50% of the experimentally introduced caterpillars on each cultivar. Irrespective of their attractiveness as determined by wind-tunnel assays, all of the cultivars sustained a fairly high proportion of parasitized caterpillars. Nonetheless, in the field intraspecific variation resulted in differential parasitism rates of herbivores. Parasitization level depended on context: when accompanied by a more attractive cultivar, as established in the laboratory study, parasitization level on a cultivar was lower than in the absence of the former. Our results contribute to the accumulating evidence that intraspecific variation in volatile emission leads to differential parasitism or predation of herbivores in the field. Recently, *Nicotiana attenuata* genotypes modified for volatile emission were shown to effectively increase herbivore egg predation by attracting generalist predators (Halitschke et al. 2008). Specialist aphid parasitoids were differentially attracted to near-isogenic lines of *Brassica oleracea*, which resulted in differential parasitization rates on aphids (Bradburne and Mithen 2000). Belowground, entomopathogenic nematodes discriminated between cultivars of maize under laboratory conditions and in the field these cultivars differed in their predation pressure on root feeding beetle larvae (Rasmann et al. 2005). Me-

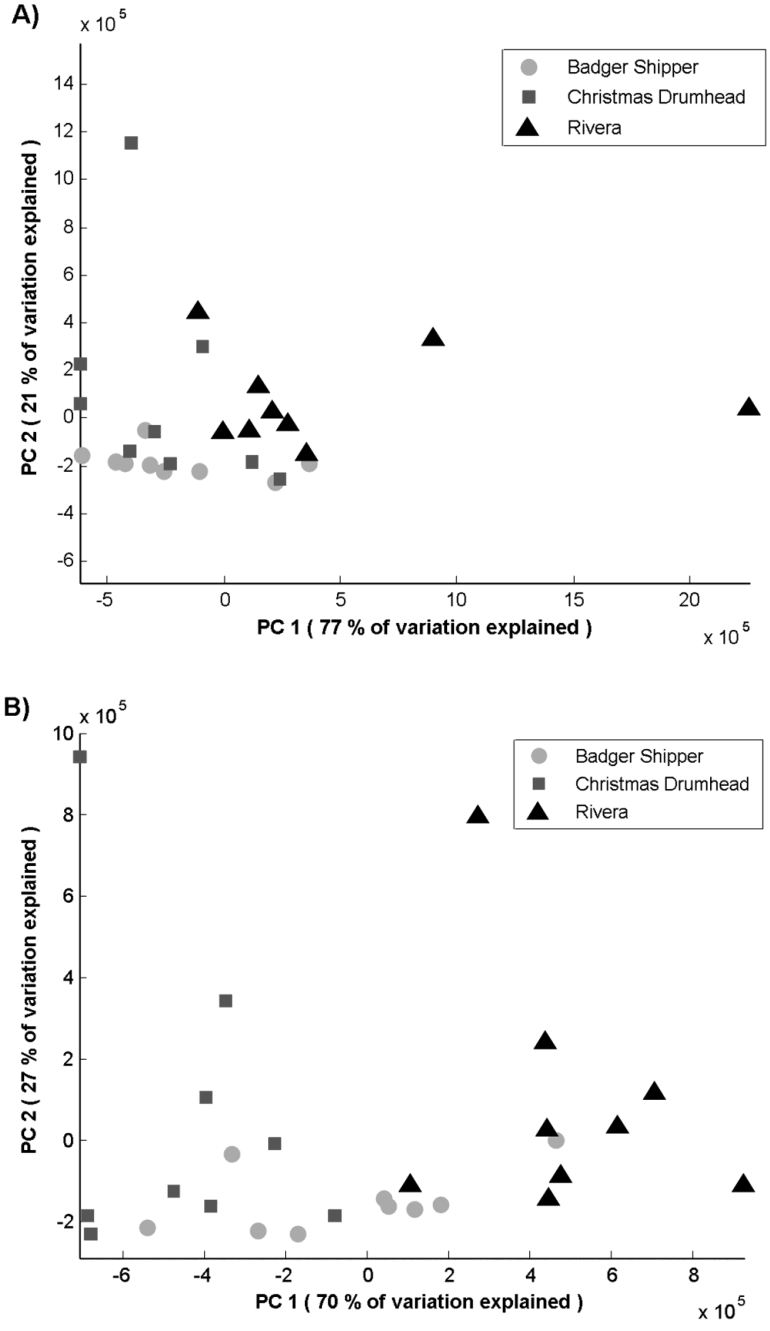
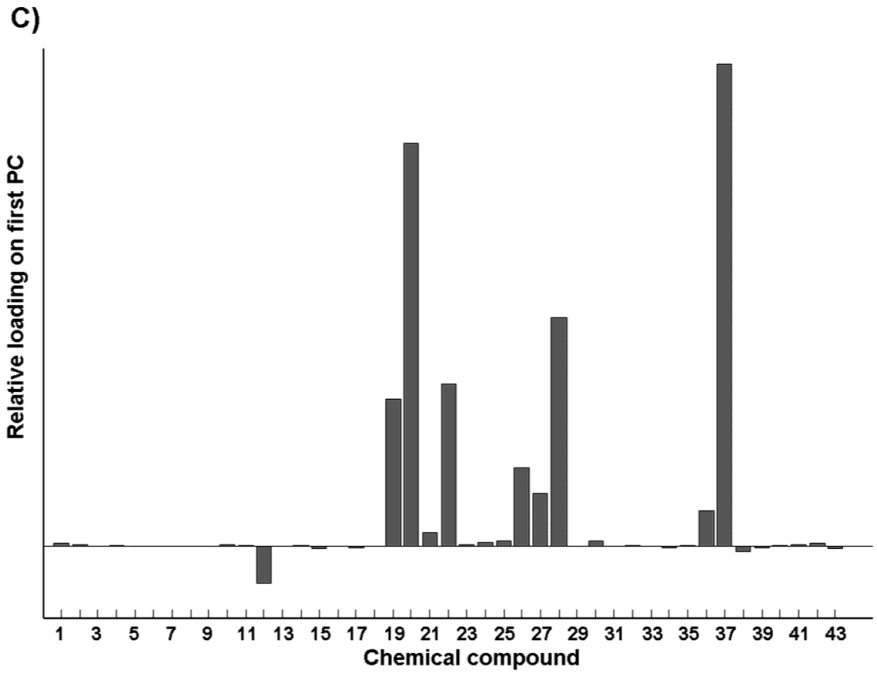


Figure 4. PCA plots of headspace composition of control (A) and *Pieris*-induced plants (B) of three *Brassica oleracea* cultivars. In both analyses separation between the cultivars was strongest on the first axis. The loadings of chemical compounds on the first Principal Component axis (C) were similar for PCA analysis of control and induced plants. The identity of the compounds depicted as numbers on the X-axis in plot C correspond with the numbers in Table 3.



chanical clipping of sagebrush, *Artemisia tridentata*, attracted more predatory coccinellid beetles (Karban 2007). However, attraction of these beetles did not lead to a reduction in plant damage. Other herbivores, such as grasshoppers and deer, which are not vulnerable to arthropod predators, were responsible for the majority of plant damage. It may, therefore, be argued that HIPVs not always contribute significantly to effective reduction of plant damage. Furthermore, HIPVs have been found to attract specialist herbivores (Halitschke et al. 2008). HIPVs are perceived by plant synergists such as parasitoids and predators but are unavoidably also perceived by herbivores, and may result in induced indirect resistance to one herbivore but induced susceptibility to others. The benefit of HIPVs to a plant is therefore likely to be context-specific for the second and higher trophic level community that a plant genotype is exposed to. Nevertheless, above- as well as belowground HIPVs do result in reduced numbers of the herbivore that is eliciting the volatile emission in the plant (Bradburne and Mithen 2000; Rasmann et al. 2005; Halitschke et al. 2008).

The attraction of parasitoids to HIPVs of plant cultivars may be fine-tuned by their previous experiences. Parasitoids may learn to associate the complex mixtures of HIPVs with the presence of hosts (Vet and Dicke 1992; Dicke and Vet 1999). However, specific compounds have also been identified to play an important role in the attraction of predators and parasitoids (de Boer and Dicke 2004; Rasmann et al. 2005; Mumm et al. 2008a, b). The HIPVs mixtures of the cultivars were relatively similar and only 43 compounds were consistently present in the replicates of headspace samples of at least a single treatment. All the terpenoids that are hypothesized to play an important role in attracting parasitoids (Dicke 1994; Heil et al. 2008) showed inconsistent emission patterns upon herbivory. In some cultivars the terpene emissions increased, whereas in other cultivars they decreased after herbivory. Only two compounds, methyl salicylate and methyl thiocyanate, were induced upon herbivory and their induced concentrations in the cultivars matched with differences in parasitoid attraction. The cultivar that was most attractive to parasitoids

Table 3. Volatile compounds detected in the headspace of three *Brassica oleracea* cultivars uninfested (control, n = 9) or infested with larvae of *Pieris rapae* (induced, n = 9). In the table the compounds are included that are present in all replicates of at least one of the cultivar/treatment combinations. Amounts of individual compounds are given as average peak area (SE) per litre of trapped air per gram shoot biomass. Bold face type indicates compounds that are identified in PCA analyses as explanatory for cultivar differences between control or induced plants, or differences among cultivars for control and induced plants.

			Christmas Drumhead	
Compound	Retention time (min)	Class	Control	Induced
1. tridecanal	33.73	Aldehyde	4.50 (1.24)	5.42 (1.60)
2. tetradecanal	39.22	Aldehyde	12.17 (2.79)	7.51 (1.99)
3. tetradecane	27.51	Alkane	2.89 (1.06)	4.09 (0.93)
4. hexadecane	38.45	Alkane	4.17 (1.13)	4.04 (0.83)
5. 2,4 dimethyl heptane	3.43	Methyl-alkane	0.60 (0.18)	1.19 (0.97)
6. benzene derivate	10.64	Benzene	0.15 (0.10)	0.84 (0.28)
7. 1,3,5 trimethyl benzene	6.88	Benzene	2.39 (0.95)	5.91 (1.33)
8. tetradecene	27.06	Alkene	0.83 (0.17)	0.94 (0.22)
9. pentadecene	32.51	Alkene	1.76 (0.26)	2.14 (0.53)
10. 1-tridecene	21.96	Alkene	6.40 (1.28)	2.47 (0.58)
11. methyl salicylate	**17.42**	**Ester**	**-**	**6.92 (1.70)**
12. 3-hexen-1-ol-acetate	**9.49**	**Ester**	**314.55 (138.45)**	**294.82 (126.72)**
13. 2,4 dimethyl furan	1.97	Furan	7.09 (1.37)	13.57 (1.54)
14. 2 methyl furan	1.42	Furan	3.17 (1.01)	2.77 (0.49)
15.5-ethyl 2(5H) furanone	7.99	Furan	3.91 (1.81)	6.78 (4.09
16. indane	10.14	Indole	0.23 (0.09)	0.53 (0.17)
17. methyl thiocyanate	**2.10**	**Thiocyanate**	**1.95 (0.33)**	**4.92 (0.96)**
18. ocimene (trans-beta)	**10.84**	**Monoterpene**	**11.34 (3.34)**	**7.94 (3.90)**
19. myrcene (beta)	**8.48**	**Monoterpene**	**117.11 (27.47)**	**77.69 (13.55)**
20. limonene	**9.95**	**Monoterpene**	**240.66 (65.21)**	**169.73 (39.25)**
21. gamma terpinene	11.19	Monoterpene	4.67 (2.26)	4.25 (1.53)
22. alpha-thujene	**6.08**	**Monoterpene**	**64.66 (18.21)**	**54.36 (16.12)**
23. alpha-terpinolene	12.53	Monoterpene	14.40 (4.48)	7.74 (2.11)
24. alpha-terpineol	16.90	Monoterpene	3.70 (2.20)	2.87 (1.48)
25. alpha-terpinene	9.53	Monoterpene	1.69 (0.76)	1.72 (0.67)
26. alpha-pinene	**6.26**	**Monoterpene**	**84.66 (16.75)**	**58.62 (9.15)**
27. 2-beta pinene	**7.57**	**Monoterpene**	**26.07 (6.91)**	**20.37 (5.61)**
28. 1,8 cineole	**10.12**	**Monoterpene**	**135.54 (44.38)**	**88.78 (23.35)**
29. 2,6-dichloro benzonitrile	24.01	Nitrile	1.92 (0.39)	2.85 (0.39)
30. 3-hexen-1-ol (cis)	4.40	Alcohol	44.17 (16.23)	19.70 (8.32)
31. 1-hexanol	4.89	Alcohol	2.15 (0.65)	2.67 (0.78)
32. methyl isobutyl ketone	2.43	Ketone	2.05 (0.74)	4.04 (0.62)
33. 4 methyl 2-heptanone	6.82	Ketone	1.00 (0.45)	3.32 (0.33)
34. 2,4 pentadione	2.84	Ketone	26.92 (7.90)	68.49 (6.84)
35. camphene	**6.77**	**Monoterpene**	**0.45 (0.21)**	**0.34 (0.12)**
36. sabinene hydrate (cis)	**11.85**	**Terpene**	**14.61 (6.27)**	**9.25 (2.86)**
37. sabinene	**7.48**	**Monoterpene**	**253.38 (71.90)**	**179.37 (51.17)**
38. alpha-farnesene	33.17	Sesquiterpene	4.06 (2.29)	24.94 (14.93)
39. unknown	37.83	Unknown	5.12 (1.73)	6.40 (2.09)
40. unknown	23.62	Unknown	2.14 (0.97)	6.56 (0.57)
41. unknown	14.18	Unknown	3.96 (2.04)	17.54 (2.48)
42. unknown	13.72	Unknown	25.71 (5.34	27.10 (3.44)
43. unknown	4.41	Unknown	4.15 (2.11)	7.53 (1.99)

Rivera		Badger Shipper	
Control	Induced	Control	Induced
4.90 (1.81)	8.30 (1.90)	6.25 (2.60)	3.11 (1.27)
13.05 (7.49)	8.83 (1.66)	10.02 (4.07)	6.22 (3.44)
2.28 (0.90)	3.89 (1.26)	3.02 (0.98)	4.34 (1.05)
3.88 (1.82)	4.77 (1.14)	2.57 (0.88)	2.24 (0.92)
0.44 (0.24)	1.11 (0.26)	0.73 (0.27)	1.04 (0.23)
0.29 (0.18)	1.00 (0.29)	0.46 (0.19)	1.12 (0.31)
3.47 (1.05)	5.28 (1.18)	4.43 (1.03)	6.62 (1.23)
0.49 (0.14)	1.24 (0.17)	0.97 (0.28)	0.60 (0.13)
1.59 (0.20)	1.76 (0.32)	1.97 (0.38)	1.47 (0.37)
3.53 (0.58)	3.77 (0.75)	6.28 (1.73)	4.64 (1.15)
0.32 (0.32)	**6.13 (1.47)**	**-**	**0.55 (0.37)**
410.77 (73.74)	**280.12 (99.40)**	**39.60 (2.00)**	**74.35 (24.75)**
8.17 (2.40)	10.86 (1.01)	9.62 (3.25)	11.79 (2.75)
3.87 (1.09)	3.10 (0.68)	4.10 (1.18)	3.43 (0.65)
8.36 (3.42)	4.59 (1.97)	1.33 (1.32)	1.84 (0.35)
0.28 (0.17)	0.56 (0.13)	0.39 (0.12)	0.53 (0.18)
2.45 (0.83)	**3.45 (0.53)**	**0.81 (0.25)**	**1.68 (0.58)**
2.97 (1.14)	**7.31 (2.20)**	**1.45 (0.62)**	**1.90 (0.66)**
236.52 (46.77)	**266.35 (17.56)**	**96.11 (26.76)**	**141.37 (26.30)**
596.13 (129.62)	**688.75 (54.62)**	**282.07 (61.99)**	**399.85 (63.36)**
15.46 (2.47)	19.71 (3.18)	8.09 (2.80)	16.91 (6.12)
239.43 (46.33)	**268.76 (19.50)**	**105.08 (23.04)**	**144.12 (23.48)**
8.25 (1.33)	9.67 (0.88)	4.32 (1.40)	6.41 (1.79)
7.26 (5.39)	3.11 (0.56)	22.00 (5.26)	36.98 (6.60)
6.44 (1.62)	7.14 (1.45)	4.46 (2.45)	7.43 (3.43)
141.61 (25.01)	**163.29 (86.91)**	**62.29 (13.37)**	**87.20 (15.53)**
78.61 (17.75)	**88.76 (6.97)**	**34.77 (7.96)**	**47.02 (6.98)**
347.08 (81.19)	**386.49 (39.77)**	**132.74 (28.90)**	**207.75 (29.54)**
19.80 (2.86)	27.91 (0.23)	1.64 (0.43)	2.09 (0.30)
53.08 (16.54)	30.43 (12.54)	3.32 (2.52)	9.94 (1.09)
1.90 (0.50)	2.23 (0.63)	1.61 (0.51)	1.98 (0.66)
2.85 (1.18)	4.64 (0.79)	2.60 (1.05)	3.95 (0.92)
1.12 (0.55)	2.75 (0.58)	2.17 (1.03)	2.77 (0.79)
36.09 (11.09)	56.76 (7.67)	51.79 (20.71)	57.01 (12.63)
2.04 (0.72)	**1.97 (0.35)**	**0.42 (0.17)**	**1.46 (0.55)**
45.73 (10.16)	**53.50 (10.59)**	**17.89 (4.98)**	**31.73 (6.57)**
789.28 (171.97)	**830.37 (44.95)**	**337.79 (74.09)**	**432.82 (68.22)**
10.86 (3.76)	15.19 (6.50)	-	-
3.80 (1.63)	3.63 (0.81)	5.56 (0.66)	3.91 (1.19)
1.84 (0.96)	6.41 (1.26)	1.15 (0.51)	4.46 (1.55)
3.81 (2.26)	14.21 (2.99)	8.84 (3.27)	14.68 (3.34)
20.68 (5.04)	30.48 (4.66)	26.94 (10.60	29.63 (6.91)
5.72 (1.78)	3.38 (1.06)	7.45 (4.39)	3.94 (1.48)

emitted highest concentrations of the two compounds. Methyl salicylate elicits an electrophysiological response in antennae of *Cotesia* parasitoids (Smid et al. 2002). Moreover, this benzenoid has been identified as an attractant for other parasitoids and predators as well (de Boer and Dicke 2004; James and Price 2004; James and Grasswitz 2005). Traps baited with synthetic methyl salicylate attracted more predators and parasitoids in the field (James and Price 2004; James and Grasswitz 2005). The occurance of methyl thiocyanate in our samples is unexpected as its parent glucosinolate, glucocapparin, was not found in our glucosinolate analyses on the same cultivars (Poelman et al. 2008c). We cannot exclude that during the process of sampling and analysis chemical reactions have taken place that led to this product, that also has been documented in other studies on *B. oleracea* (Geervliet et al. 1997; Pinto et al. 2007). In the field, near-isogenic lines of *Brassica oleracea* differing in a gene which alters the structure of isothiocyanates were shown to differentially attract aphid parasitoids; enhanced isothiocyanate production led to increased attraction of parasitoids (Bradburne and Mithen 2000). In contrast, laboratory studies on *Pieris rapae*-infested *Arabidopsis thaliana* plants that were genetically modified to release nitriles instead of isothiocyanates, showed that *Cotesia* parasitoids preferred plants with lower isothiocyanate production (Mumm et al. 2008b). The difference in induced concentrations of methyl salicylate and methyl thiocyanate in accessions of *Brassica oleracea* that corresponds with enhanced attraction of parasitoids in our study supports a role for these compounds in indirect resistance of Brassicaceae. On the other hand, the production of methyl thiocyanate as a signal to parasitoid wasps may encompass a signal of poor offspring performance to parasitoids. High concentrations of the glucosinolate breakdown product methyl thiocyanate in the HIPV blend, might be associated with higher concentrations of the intact glucosinolate precursor. High concentrations of glucosinolates may hamper herbivore growth and affect the performance of the parasitoid larvae in the herbivore (Turlings and Benrey 1998; Gols et al. 2008a). Here we show that wasp performance in terms of the number of days until adults emerge from cocoons depends on the cultivar on which their herbivore host is feeding. Even though the most attractive cultivar, Christmas Drumhead, sustained fastest wasp development, the least attractive cultivar Badger Shipper also sustained relatively fast wasp development. This shows that a parasitoid response to high concentrations of methyl thiocyanate does not directly translate into poor larval performance. However, our results do indicate that the response of parasitoids to HIPVs not necessarily results in maximal fitness of individual offspring. Response to any cue that enhances host location may be more important to female parasitoids in maximizing fitness than discriminating against cues that result in suboptimal performance of individual offspring.

Our study shows that intraspecific variation in HIPVs of plants indeed results in differential parasitism of caterpillars in the field. Even under the complex abiotic and biotic conditions in agricultural and natural fields that may hamper host searching by parasitoids, plant cultivars were shown to differentially attract parasitoid wasps. Our work confirms that the widely used laboratory assays on HIPV-preferences of parasitoids do provide reliable information on relative parasitism differences of herbivores in the field. However, it remains to be identified whether HIPVs that enhance parasitism attraction to a plant indeed results in a fitness benefit to the plant under natural ecosystem complexity.

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Chapter 5

Picture: Tibor Bukovinszky

Plant variation in herbivore-induced volatiles affects hyperparasitoid attraction and hyperparasitoid community composition

Erik H. Poelman, Aline E. Boursault, Yde Jongema, Joop J.A. van Loon, Jeffrey A. Harvey, Louise E.M. Vet & Marcel Dicke

Abstract

Intraspecific variation in herbivore-induced plant volatile production affects the abundance of primary parasitoids and extends to hyperparasitoid communities. First, variation in the abundance of primary parasitoids may cascade higher up the food chain and thus affect hyperparasitoid abundance and diversity. Secondly, hyperparasitoids may respond directly to herbivore-induced plant volatiles. In the field, cultivars of *Brassica oleracea* that were more attractive to primary parasitoids, i.e. the solitary endoparasitoid *Cotesia rubecula* and the gregarious *C. glomerata*, harboured more hyperparasitoids than less attractive cultivars. Two of those hyperparasitoid species were identified to have the same in-flight preference in a windtunnel for cultivars of *B. oleracea* as the two primary parasitoids. Cocoons of the the gregarious *C. glomerata* were more heavily attacked by hyperparasitoids than cocoons of the solitary *C. rubecula*. Furthermore, variation among plant cultivars in herbivory-induced volatiles more strongly affected the abundance of hyperparasitoids on cocoons of the gregarious primary parasitoid than on cocoons of the solitary primary parasitoid. Herbivore-induced plant volatiles therefore not only play a role in structuring plant associated-insect communities, but they may also influence selection against gregariousness of primary parasitoids.

Key words: indirect defence, *Cotesia glomerata*, *Cotesia rubecula*, *Lysibia nana*, *Pteromalus semotus*

Introduction

Insect communities associated with plants have been found to widely differ among plant genotypes within a plant species (Johnson and Agrawal 2005; Whitham et al. 2006). Intraspecific variation in morphology or phytochemistry directly affects the abundance and diversity of herbivorous insects (Johnson and Agrawal 2005; Bangert et al. 2006; Wimp et al. 2007; Poelman et al. 2008a) and this may cascade into effects on higher trophic levels. The abundance and diversity of organisms in the third trophic level, such as predatory and parasitic arthropods, is often positively correlated with herbivore abundance and nutritional quality (Müller and Godfray 1999; Bailey et al. 2006; Bukovinszky et al. 2008). Similarly, the abundance and quality of organisms at the third trophic level (primary parasitoids) influences the performance and efficacy of organisms in the fourth trophic level, such as hyperparasitoids that parasitize primary parasitoids (Harvey et al. 2003; Buitenhuis et al. 2005; Bukovinszky et al. 2008).

The abundance of organisms at the third trophic level may also be promoted through a variety of indirect mechanisms. Plants can offer natural enemies of their own attackers housing or food sources such as extrafloral nectar to enhance the local abundance of bodyguards that attack herbivorous arthropods (Heil 2008). This may affect third trophic level diversity independent of the effects mediated through the second trophic level. Herbivore-induced plant volatiles that attract predators and parasitoids are a well-known example of a plant trait that promotes the abundance and diversity of parasitoids (Heil 2008). The changes in volatile production by a plant, as elicited by herbivore attack, attract parasitoids that attack the herbivore and thereby indirectly defend plants against its attackers (Turlings et al. 1995; Takabayashi and Dicke 1996; De Moraes et al. 1998). However, hyperparasitoids that attack primary parasitoids, including secondary species that attack the cocoons of the primary parasitoid, may also use herbivore-induced plant volatiles to locate their hosts (Sullivan and Völkl 1999). The cocoons themselves may be inconspicuous and, therefore, wasps may use herbivore-induced plant volatiles elicited by parasitized herbivores as a cue to locate their host (Vet and Dicke 1992; Vet et al. 1995; Völkl and Sullivan 2000; Arimura et al. 2005). Parasitized herbivores have been shown to induce different volatile blends in plants compared with healthy caterpillars (Fatouros et al. 2005) and plant-derived cues may thus provide hyperparasitoids with a reliable signal of the presence of their host (Sullivan and Völkl 1999). Plant cultivars have been identified to differ substantially in their volatile production and resulting attractiveness to primary parasitoids and predators (Geervliet et al. 1997; Krips et al. 2001; Poelman et al. 2008d). However, the role of herbivore-induced plant volatiles in structuring parasitoid communities up to the fourth trophic level has to our knowledge not been explored (Bruinsma and Dicke 2008; Poelman et al. 2008e).

Here we studied whether hyperparasitoid communities of two primary parasitoids from the genus *Cotesia* (Hymenoptera: Braconidae) are affected by plant cultivar-related differences in herbivore-induced volatile production (Figure 1). We used four cultivars of *Brassica oleracea* that we identified in a previous study to differ in attractiveness to *Cotesia glomerata* and *Cotesia rubecula* (Poelman et al. 2008d). The two primary parasitoids attack *Pieris* caterpillars. *Cotesia glomerata* is a gregarious primary endoparasitoid that accepts a range of *Pieris* hosts, but prefers *P. brassicae* (Brodeur et al. 1998). *Cotesia rubecula* is a solitary endoparasitoid that is specialized on *Pieris rapae* (Brodeur et al. 1998). We addressed the following questions: 1) whether cocoons of *Cotesia* spp. collected from *B. oleracea* cultivars that differ in the attractiveness to primary parasitoids differ in the degree of hyperparasitism, 2) whether hyperparasitoids respond to and have similar in-flight preferences for herbivore-induced plant volatiles as their primary parasitoid hosts,

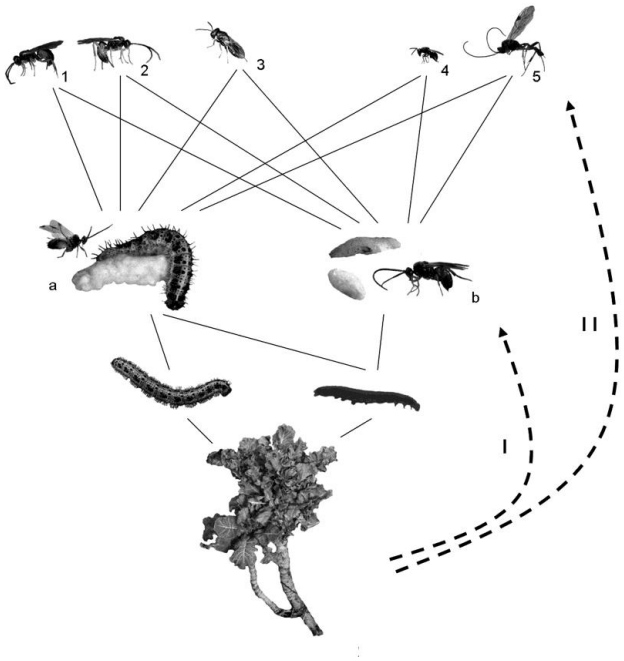

Figure 1. Effect of herbivore-induced plant volatiles on the composition of a plant associated parasitoid community. Intraspecific plant variation in herbivore-induced volatiles differentially attracts primary parasitoids of *Pieris* caterpillars (I), which may cascade into effects on hyperparasitoids and, in addition, volatiles may directly affect hyperparasitoids (II). These effects may differ between the gregarious primary endoparasitoid *Cotesia glomerata* (a) and the solitary primary parasitoid *Cotesia rubecula* (b). Both parasitoids share common hyperparasitoids: *Lysibia nana* (1), *Acrolyta nens* (2), *Pteromalus semotus* (3), *Baryscapus galactopus* (4), and *Mesochorus gemellus* (5).

and 3) whether cocoons of gregarious parasitoid species, *C. glomerata*, are more susceptible to hyperparasitism than cocoons of the solitary parasitoid species, *C. rubecula* (Figure 1). In field experiments performed in the spring and summer seasons over three consecutive years we collected cocoons of the solitary and gregarious parasitoids from different *B. oleracea* cultivars. Wasps emerging from the cocoons were identified to species level and the hyperparasitoid communities for each of the plant cultivars and primary parasitoids were compared. Under controlled laboratory conditions we tested the in-flight preference of two hyperparasitoid species for herbivore-induced volatiles of two *B. oleracea* cultivars.

The gregarious parasitoid *C. glomerata* was found to be more commonly hyperparasitized than the solitary parasitoid *C. rubecula* on each of the plant cultivars that differed in attractiveness to primary parasitoids. Differences in herbivore-induced plant volatile production by the plants especially affected the number of gregarious parasitoid cocoon clutches parasitized by a hyperparasitoid. The plant cultivar that was most attractive to primary parasitoids in the field also sustained the highest proportion of primary parasitoid broods that were containing a hyperparasitoid. This observation was in concordance with the in-flight preference of hyperparasitoids as assessed in a Y-tube olfactometer in the laboratory. We discuss the role of herbivore-induced plant volatiles in structuring the plant-associated insect community and how plant volatile production may consequently impose selection on the life history of primary parasitoids.

Material and Methods

Plants

We used four white cabbage cultivars (*Brassica oleracea* L.var. *alba*) that were identified in our previous work to differ in attractiveness to the primary parasitoids *Cotesia glomerata* and *Cotesia rubecula* (Poelman et al. 2008d). When infested with *Pieris* caterpillars, the cultivar Christmas Drumhead (Centre for Genetic Resources, CGN-Wageningen, The Netherlands) is more attractive than Rivera (Bejo Zaden BV, Warmenhuizen, The Nether-

lands) that is in turn more attractive than the cultivars Badger Shipper and Lennox (CGN-Wageningen and Bejo Zaden respectively) (Poelman et al. 2008d). Seeds of plants used for olfactometer experiments were germinated on peat soil and seedlings were transferred to 1.45-l pots containing peat soil (Lentse potgrond, no. 4, Lent, The Netherlands). Plants were placed in a glasshouse compartment (18-26 °C, 50-70% r.h.), provided with SON-T light (500 µmol/m2/s; L16:D8) in addition to daylight, and when plants were four weeks old they were fertilized every other week by applying 100 ml nutrient solution of 2.5 mg/l Kristalon Blauw ((Hydro Agri Rotterdam, The Netherlands) (N-P-K-Mg) 19-6-20-3)) to the soil. We used seven-week-old plants in olfactometer experiments. Seeds of plants used in common garden experiments were directly sown into peat soil cubes. Trays with soil cubes containing three-week-old seedlings were placed outside during the day to adapt the plants to field conditions. When they were five weeks old, plants were transplanted with their soil cubes into the soil at the experimental site.

Insects
Stock colonies of *Cotesia glomerata* were maintained on the host *Pieris brassicae* feeding on Brussels sprouts plants (*Brassica oleracea* L. var. *gemmifera*) in a greenhouse (22-24 ° C, 50-70% r.h., 16L:8D photoperiod). *Pieris brassicae* caterpillars are parasitized by *C. glomerata* in the first (L1) to third (L3) instar and *C. glomerata* larvae leave their host to spin a cocoon when the caterpillar host is in the fifth (= terminal) larval instar. The secondary hyperparasitoids *Lysibia nana* and *Pteromalus semotus* were reared on cocoons of *Cotesia glomerata* that were kept in a climate cabinet (22-24 °C, 40-70% r.h., 16L:8D photoperiod).

Common garden experiment
In an agricultural field in the vicinity of Wageningen, The Netherlands, we conducted common garden experiments in three consecutive years (2005-2007). Each year in week 19 (early May), we established plots of 6 x 6 m containing a monoculture of 49 plants of one of the four cultivars. Within plots, plants were planted in a square of 7 x 7 with a spacing of 75 cm between plants. Plots were isolated by a strip of 6 meters that was sown with a grass mixture of *Lolium* and *Poa* species. In 2005 we established eight plots of the four cultivars using a randomized design. In 2006 and 2007 we used a subset of these cultivars to focus on differences between only two cultivars. Those cultivars were shown in the laboratory to differ in attractiveness to *Cotesia* parasitoids, but this difference in attraction was not expressed in the field when placed together with an even more attractive cultivar (Poelman et al. 2008d). In 2006 we established 20 plots of the cultivars Rivera and Badger Shipper, whereas in 2007 we studied the comparison between Christmas Drumhead and Rivera by establishing 18 plots for both cultivars.

Collection of cocoons
During the growth season of cabbage plants, from early May to the end of September, we conducted weekly surveys on the plants for *Cotesia glomerata* and *C. rubecula* cocoons by investigating both sides of all their leaves. The cocoons were collected and placed individually in 2.2 ml Eppendorf tubes, closed with cotton wool and labeled for the plot they originated from. The cocoons were weighed and for the gregarious *C. glomerata* the brood size was assessed by counting the number of cocoons in a clutch. The Eppendorf tubes were checked daily for emerging parasitoids, which were transferred to another Eppendorf tube and stored at -20 ºC. All wasps were identified to species level.

Y-tube olfactometer

The olfactory response of two hyperparasitoids, i.e. *Lysibia nana* (Hymenoptera: Ichneumonidae) and *Pteromalus semotus* (Hymenoptera: Braconidae), to volatiles emitted by *Pieris*-infested plants of different *B. oleracea* cultivars was tested in a Y-tube olfactometer (Takabayashi and Dicke 1992). Hyperparasitoids that were reared without exposure to plant odours and without oviposition experience were offered two-choice tests between Christmas Drumhead (attractive to *C. glomerata* and *C. rubecula*) and Badger Shipper (less attractive to *C. glomerata* and *C. rubecula*). Plants were infested with two fifth instar parasitized *Pieris brassicae* caterpillars 24 hours before the experiment. At the start of the experiment the plants were removed from their pots, excessive soil around the roots was removed and the belowground plant part was packed in aluminum foil. A single plant per cultivar with the caterpillars on it was placed in one of the two glass jars (30 l) that were connected to the two olfactometer arms. The airflow (4 l/min) through the jars was first filtered over charcoal and then led through the two jars each containing a plant from a different cultivar. At the junction of the two olfactometer arms the odours of the two jars were mixed to yield an air stream with a flow rate of 8 l/min that passed further down the stem section (3.5 cm diameter, 22 cm length) to the release point of the wasps. At the release point the air was extracted by a vacuum pump at 8 l/min. A single wasp was released in each test. Wasps that passed a set line at the end of one of the olfactometer arms within 10 minutes and stayed there for at least 15 seconds were considered to have chosen for the odour released by the cultivar attached to that olfactometer arm. Wasps that did not make a choice within 10 minutes were considered as non-responding individuals. To compensate for unforeseen asymmetry in the setup we swapped the jars containing the plants after testing five wasps and replaced the set of plants after testing ten wasps. The Y-tube olfactometer setup was placed in a climatized room and in addition to daylight it was illuminated with 4 fluorescent tube lights (FTD 32 W/84 HF, Pope, The Netherlands) that were positioned 90 cm above the setup.

Statistical analysis

First, we tested whether solitary and gregarious cocoons of the primary parasitoids collected from the four cultivars differed in the proportion of occasions that these were found by a hyperparasitoid. For each solitary cocoon and each gregarious cocoon clutch, both

Table 1. Hyperparasitoid species and the number of hyperparasitoid wasps emerging from *Cotesia glomerata* and *Cotesia rubecula* cocoons collected during a three year survey.
[1] Gregarious parasitoid, numbers indicate the number of *Cotesia* cocoons that were parasitized, containing on average eight *B. galactopus*
[2] Number indicates the collected number of cocoon clutches

Hyper parasitoid species	Family	Sub family	Parasitism mode	*Cotesia glomerata*[2] (n =1256)	*Cotesia rubecula* (n = 1668)
Acrolyta nens	Ichneumonidae	Cryptinae	Secondary	313	1
Lysibia nana	Ichneumonidae	Cryptinae	Secondary	3662	8
Gelis agilis	Ichneumonidae	Cryptinae	Secondary	12	1
Gelis spec.	Ichneumonidae	Cryptinae	Secondary	1	-
Bathythrix aerea	Ichneumonidae	Cryptinae	Secondary	1	-
Mesochorus gemellus	Ichneumonidae	Mesochorinae	Primary	198	82
Pteromalus semotus	Braconidae	Hexothecinae	Secondary	536	28
Pteromalus chrysos	Braconidae	Hexothecinae	Secondary	14	-
Baryscapus galactopus[1]	Eulophidae	Tetrastichinae	Primary	1722	79

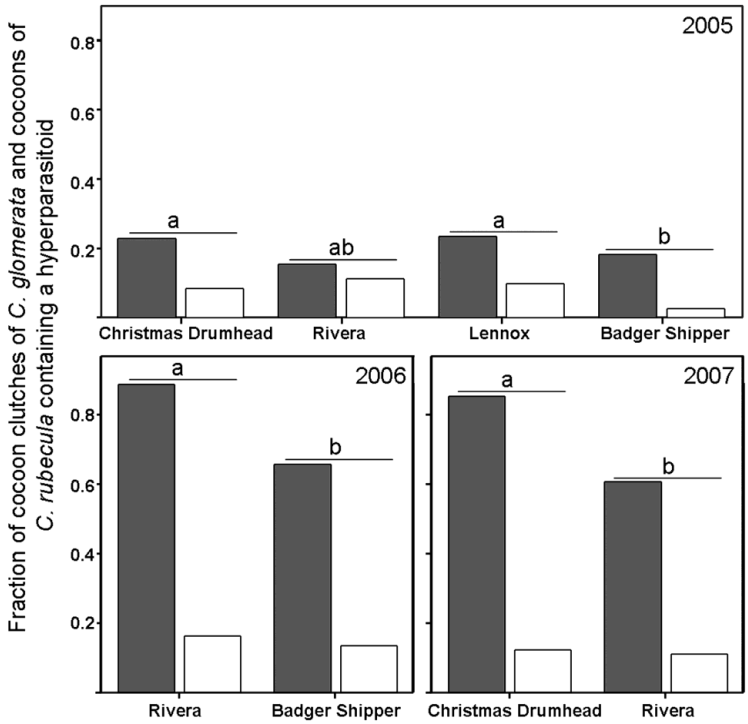

Figure 2. Fraction of cocoon clutches of the gregarious *Cotesia glomerata* (grey bars) and cocoons of the solitary *C. rubecula* (white bars) collected from *Brassica oleracea* cultivars from which at least a single hyperparasitoid wasp emerged. The bars thus indicate the chance that a solitary cocoon or clutch of cocoons is found by a single hyperparasitoid wasp on a particular cultivar. The cultivars are ordered from left to right by decreasing attractiveness to the primary parasitoids of the genus *Cotesia* as identified in a previous study (Poelman et al. 2008d). Letters above the bars indicate post hoc differences between cultivars at the level of $P < 0.05$.

consisting of parasitoid larvae that emerged from a single caterpillar, we scored a 1 when there was any hyperparasitoid emerging and a 0 when there were only primary parasitoids emerging. We used a Generalized Linear Model (GLM) to test for the effect of the fixed factors cultivar and *Cotesia* species as well as their interaction on the binomially distributed occurrence of hyperparasitism. Secondly, we tested whether differences in the proportion of clutches found by a hyperparasitoid also resulted in differences in the composition of parasitoid communities. Within each of the years, for each cultivar and *Cotesia* species we counted the total number of emerging wasps for each of the primary and hyperparasitoid species. One of the hyperparasitoid species, *Baryscapus galactopus*, is a primary species that develops gregariously and emerges from *Cotesia* cocoons (M.R. Shaw, personal communication). We recalculated their total numbers to the number of individual *Cotesia* cocoons that was parasitized by this species by dividing the observed number by 8 and rounding this figure off to the nearest whole number. The division by 8 was based on the average number of *B. galactopus* found in a single *Cotesia rubecula* cocoon. The data on species composition and their abundance per *Cotesia* species were subjected to chi square tests to test for parasitoid community differences on cultivars. All statistical tests were performed with the statistical software package Gen Stat (10th edition).

Results

During the three years of this study we collected 1668 cocoons of the solitary primary parasitoid *C. rubecula* and 1256 cocoon clutches of the gregarious primary parasitoid *C. glomerata*. We found that the primary parasitoids were parasitized by nine hyperparasitoid species (Table 1). The proportion of *C. glomerata* cocoon clutches from which any hyperparasitoid emerged was higher than the proportion of hyperparasitoids emerging from the

Table 2. The effect of cultivars and *Cotesia* species on the fraction of hyperparasitism in field experiments carried out in three consecutive years. Boldface type present significant effects at $P < 0.05$ in a GLM model with a binomial distribution.
[1] In 2005 d. f. of cultivar is 3 in 2006-2007 d. f. = 1

	Full model	Cultivar[1]		*Cotesia* species		Cultivar * *Cotesia*	
	deviance	deviance	*P*	deviance	*P*	deviance	*P*
2005	902.09	7.81	**0.048**	43.54	< **0.001**	13.27	**0.004**
2006	1278.48	4.87	**0.027**	409.07	< **0.001**	1.75	0.186
2007	909.31	10.02	**0.002**	183.79	< **0.001**	1.05	0.306

single cocoons of the solitary *C. rubecula* (Figure 2, Table 2), meaning that gregarious clutches are more heavily attacked by hyperparasitoids than single cocoons. Furthermore, individual broods of *C. glomerata* were parasitized by between one and four species of hyperparasitoids, showing that our statistical analysis underestimates the relative exposure of gregarious clutches to hyperparasitoids. Within each year, the proportion of solitary cocoons of *C. rubecula* and cocoon clutches of *C. glomerata* that were attacked by at least a single hyperparasitoid differed among the cultivars from which the cocoons had been collected (Figure 2, Table 2). In 2005 the cultivars that were most attractive to primary parasitoids (both *Cotesia* species), i.e. Christmas Drumhead and Rivera, had higher proportions of cocoons and cocoon clutches containing at least a single hyperparasitoid than those found on Badger Shipper. However, the differences between cultivars were relatively small due to low overall rates of hyperparasitism on *C. glomerata* in 2005 (Figure 2, Table 2). In 2006, a pair-wise comparison between Rivera and Badger Shipper showed that again solitary cocoons and cocoon clutches collected on the more attractive Rivera were hyperparasitized more frequently than those collected on Badger Shipper (Figure 2, Table 2). Similarly, in 2007 a comparison between Christmas Drumhead and the less attractive Rivera revealed that cocoons and cocoon clutches that originated from Christmas Drumhead plants were more frequently hyperparasitized than those found on Rivera (Figure 2, Table 2).

Cocoon clutches of *C. glomerata* that were attacked by hyperparasitoids had average hyperparasitization percentages of 65 to 81 % of the individual cocoons in the clutch, resulting in overall hyperparasitism of individual cocoons ranging from 20 to 55% in the three years (Figure 3). Over the three years individual cocoons of *C. rubecula* were hyperparasitized on average 5 to 15% of the cases. Hyperparasitoid species contributing most to hyperparasitism differed between the two *Cotesia* species. *Lysibia nana* and *Baryscapus galactopus* were the most abundant hyperparasitoids of *C. glomerata*, whereas *Mesochorus gemellus* and *B. galactopus* were the most abundant hyperparasitoids of *C. rubecula* (Table 1, Figure 3). Furthermore, the higher fraction of *C. glomerata* cocoon clutches and *C. rubecula* cocoons found by hyperparasitoids on cultivars that are attractive to primary

Table 3. Chi square tests for the community composition of parasitoids associated with cocoons of two *Cotesia* wasps and the effect of the *B. oleracea* cultivar on parasitoid community composition in three consecutive years (2005-2007). Boldface type present significant effects at $P < 0.05$

	Cotesia glomerata			*Cotesia rubecula*		
	d. f.	χ^2	*P*	d. f.	χ^2	*P*
2005	24	213.0	< **0.001**	9	19.3	**0.023**
2006	8	24.6	**0.002**	4	15.3	**0.004**
2007	8	276.0	< **0.001**	4	5.8	0.21

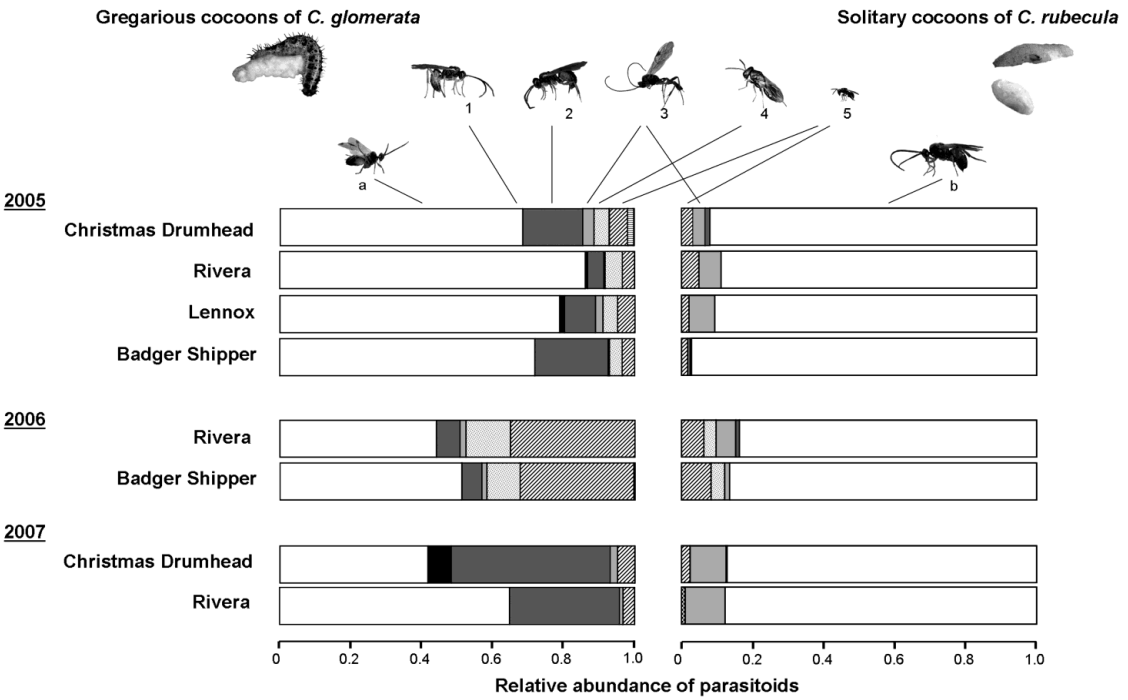

Figure 3. Relative abundance of the primary parasitoids and hyperparasitoids reared from cocoons of the gregarious *Cotesia glomerata* (left) and solitary *Cotesia rubecula* (right) that had been collected from four *Brassica oleracea* cultivars during three field seasons. Colours and patterns indicate the different parasitoid species; white bars depict the primary parasitoids (a) *Cotesia glomerata*, (b) *Cotesia rubecula;* coloured bar segments represent the most abundant hyperparasitoids: *Acrolyta nens* (1, black bar), *Lysibia nana* (2, dark grey bar), *Mesochorus gemellus* (3, light grey), *Pteromalus semotus* (4, stippled bar), *Baryscapus galactopus* (5, striped bar). Within each year the cultivar that is most attractive to primary parasitoids also harbours a more diverse hyperparasitoid community.

parasitoids also resulted in a different plant-associated hyperparasitoid community (Figure 3, Table 3). Individual cocoons of the *Cotesia* species collected on cultivars that are attractive to primary parasitoids contained relatively more hyperparasitoid wasps that were often members of more different species than found in cocoons collected from cultivars that are less attractive to the primary parasitoids (Figure 3, Table 3).

Volatiles induced in Christmas Drumhead plants by parasitized fifth instar caterpillars were more attractive to naïve females of the hyperparasitoids *Lysibia nana* and *Pteromalus semotus* than herbivory-induced volatiles of Badger Shipper plants (binomial test *L. nana*: $P < 0.001$; *P. semotus*: $P = 0.014$). Thus, a cultivar that is more attractive to primary parasitoids of the genus *Cotesia* is also more attractive to (at least some of) their hyperparasitoids.

Discussion

Within each of the study years, cocoons of primary parasitoids collected on *B. oleracea* cultivars that were more attractive to primary parasitoids (Poelman et al. 2008d) were more commonly hyperparasitized than cocoons collected on cultivars that are less attractive to primary parasitoids. Consequently, hyperparasitoids of several species constituted a larger share of the parasitoid community on cultivars that were attractive to primary parasitoids.

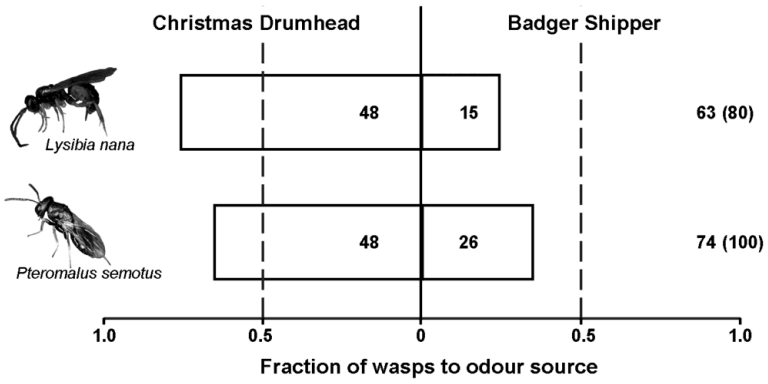

Figure 4. Preference of *Lysibia nana* and *Pteromalus semotus* for the volatiles of *Brassica oleracea* cultivars (Christmas Drumhead and Badger Shipper) infested with L5 *Pieris brassicae* caterpillars that are parasitized by *Cotesia glomerata*. The numbers in the bars indicate the number of wasps that made a choice for the odour of a particular cultivar in a two-choice Y-tube olfactometer. The numbers on the right of each bar represent the number of wasps that made a choice and between brackets the total number of wasps tested.

The in-flight preference of two hyperparasitoid species for herbivore-induced plant volatiles of the cultivar that is more attractive to primary parasitoids shows that intraspecific plant variation in herbivore-induced volatile emission affects the composition of parasitoids at the fourth trophic level associated with the plant.

Our study provides experimental evidence that hyperparsitoids associated with caterpillar-primary parasitoid complexes use plant derived cues in host searching. Intraspecific plant variation in volatiles induced by late-instar parasitized *P. brassicae* caterpillars resulted in differential attraction of hyperparasitoids to plant cultivars. The hyperparasitoids may use plant-derived cues elicited by parasitized caterpillars since the cocoons of primary parasitoids are relatively inconspicuous. The use of host plant cues by hyperparasitoids has been studied previously in aphid hyperparasitoids. Few studies have thus far identified that plant-derived cues affected host searching by aphid hyperparasitoids (Singh and Srivastava 1987; Völkl and Sullivan 2000) and other studies have not yet demonstrated that hyperparasitoids orient their flight to plant odours (Read et al. 1970; Buitenhuis et al. 2005). Wind dispersal may hamper active volatile-oriented flights by aphid hyperparasitoids, because of their small size. Nonetheless, aphid hyperparasitoids are known to be arrested by cues that are an indication of the presence of their hosts, such as honeydew, aphids and primary parasitoids (Budenberg 1990; Sullivan and Völkl 1999; Buitenhuis et al. 2005). When secondary hyperparasitoids associated with large herbivores, such as the macro-Lepidoptera, arrive on a host plant possibly by detecting herbivore-induced volatiles, these wasps may also become arrested by cues that indicate the presence of their host -cocoons. Alongside density- and trait-mediated effects that cascade through herbivores (Bukovinsky et al. 2008), plant variation in herbivore-induced volatiles can structure the diversity of hyperparasitoid communities independent of effects on herbivores. Furthermore, the attraction and arrestment of hyperparasitoids make plants an enemy-dense space for primary parasitoids. This may result in rapid patch leaving tendencies of primary parasitoids when they encounter hyperparasitoids or cues indicating their presence (Höller et al. 1994). The behavioural response of the primary parasitoid to hyperparasitoid presence may thereby result in a reduction of parasitism rates of herbivores on the plant. Hyperparasitoid responses to herbivore induced plant volatiles may thus form a counter-selective force against the induced indirect resistance of plants that consists of the attraction of primary parasitoids.

Consequences for a gregarious primary parasitoid

Cocoon clutches of the gregarious *C. glomerata* were not only more heavily attacked by hyperparasitoids, but also the total number of individual cocoons that were hyperparasitized was higher than cocoons of the solitary *C. rubecula*. Although both primary parasitoids were hyperparasitized by several of the same species, cocoons of *C. glomerata* were more susceptible to some hyperparasitoid species than were cocoons of *C. rubecula*. However, in contrast with our results, *C. rubecula* suffered higher hyperparasitism than *C. glomerata* in a study in the U.S.A. (McDonald and Kok 1991). This could be due to the fact that one of the most dominant hyperparasitoids of *Cotesia* species in Eurasia, *Lysibia nana*, is absent in the Nearctic (McDonald and Kok 1991; Gaines and Kok 1999). During close-range searching, gregarious cocoons may be more conspicuous to hyperparasitoids than solitary cocoons due to the larger overall size and consequently higher density of cues associated with a clutch of cocoons compared to individual cocoons. Furthermore, hyperparasitoids may be arrested after finding a gregarious brood because of the reward of several cocoons. Depending on their egg load the hyperparasitoids may readily parasitize all cocoons or a large part of the brood even when they are egg-limited (Hoffmeister et al. 2005 for this theory in patch use by primary parasitoids; Harvey 2008). For example, the dynamics of egg maturation in *L. nana* and another common secondary hyperparasitoid of *Cotesia* species, *A. nens*, closely approximate the average brood size (e.g. 20-40) of *C. glomerata*, suggesting that these hyperparasitoids have co-evolved with this primary parasitoid (Schwarz and Shaw 2000; Harvey 2008; Harvey et al. 2008). In situations where a female hyperparasitoid finds a gregarious brood but has insufficient eggs available to parasitize the brood over the course of a few hours, the female wasp may extend her temporal duration of brood attending or may even guard the host brood against other females until she has fully exploited it (Goubault et al. 2007).

Furthermore, we found that two hyperparasitoids, including *Lysibia nana*, oriented their host searching to herbivore-induced plant volatiles of the cultivar that is attractive to primary parasitoids above a cultivar that is less attractive to primary parasitoids. In the field, the gregarious parasitoid *C. glomerata* was more profoundly affected by cultivar differences in hyperparasitoid pressure than the solitary *C. rubecula*. Plant cultivar differences in herbivore-induced volatile emission may thus lead to a larger hyperparasitism risk for gregarious than for solitary primary parasitoids. Herbivore-induced plant volatiles may thus increase selection pressure against large brood sizes of primary parasitoids.

The debate on the evolution of clutch size evolution has originally been postulated to be a manifestation of parent-offspring conflict (Godfray and Parker 1992). The fitness of individual offspring within a brood is generally negatively correlated with increasing brood size, whereas parental fitness increases with larger brood size. Therefore, the optimum brood size for parents is often larger than that for the individual offspring. Parents are found to adjust their brood size to host resources, optimizing the quantity and quality of their offspring (Le Masurier 1987). On the other hand, individual offspring are under selection to increase their own fitness, which may favour the killing of competitors (Godfray and Parker 1992; Pexton et al. 2003; Pexton and Mayhew 2004). Alongside parent-offspring conflicts over brood size, our data show that hyperparasitoids can impose strong frequency-dependent selection on brood size of primary parasitoids. Furthermore, pressure on primary parasitoids from their own natural enemies, including predators and hyperparasitoids, may have resulted in the evolution of usurpation of the herbivore host by parasitoids to reduce hyperparasitism (Brodeur and Vet 1994). The ecology of healthy insects is often significantly different from parasitized conspecifics, which has been sug-

gested as an adaptive form of regulation by the parasitoid reducing mortality of the parasitoid brood during the time it is susceptible to attack from predators and hyperparasitoids (Fritz 1982; Brodeur and McNeil 1989). Although cocoons of wasps that usurped their herbivorous host to move to places where wasp cocoons were more concealed, after the parasitoid larvae egress from the host and spin cocoons are unprotected and vulnerable. However, some gregarious parasitoids, including *C. glomerata*, manipulate their herbivorous host post-larval in such a way that the herbivore may guard the parasitoid cocoons. In some species emerged larvae increased their survival by attaching their cocoons to the still mobile herbivore, whereas other species such as *C. glomerata* attach their cocoons to immobile herbivores that display aggressive behaviour when they are disturbed (Kester and Jackson 1996; Tanaka and Ohsaki 2006; Harvey et al. 2008; Grosman et al. 2008). Post-larval emergence usurpation strategies have only been reported from gregarious parasitoids, which may suggest that hyperparasitoids impose a stronger selection on gregarious parasitoids than on solitary parasitoids.

Plant genotypic variation in herbivore-induced responses affects the plant-associated hyperparasitoid community. Alongside plant traits that structure insect communities as mediated through effects on herbivores, the composition of higher trophic levels is directly affected by plant traits involved in indirect resistance. Herbivore-induced plant volatiles most profoundly affect the hyperparasitoid pressure on gregarious cocoons. Gregarious parasitoids responding to herbivore-induced plant volatiles may thereby be selected for strategies that reduce the exposure to hyperparasitoids. These include usurpation strategies or changes in brood size. The attraction of hyperparasitoids to herbivore-induced plant volatiles may catalyze natural selection for life-history tactics with small brood size in primary parasitoid wasps.

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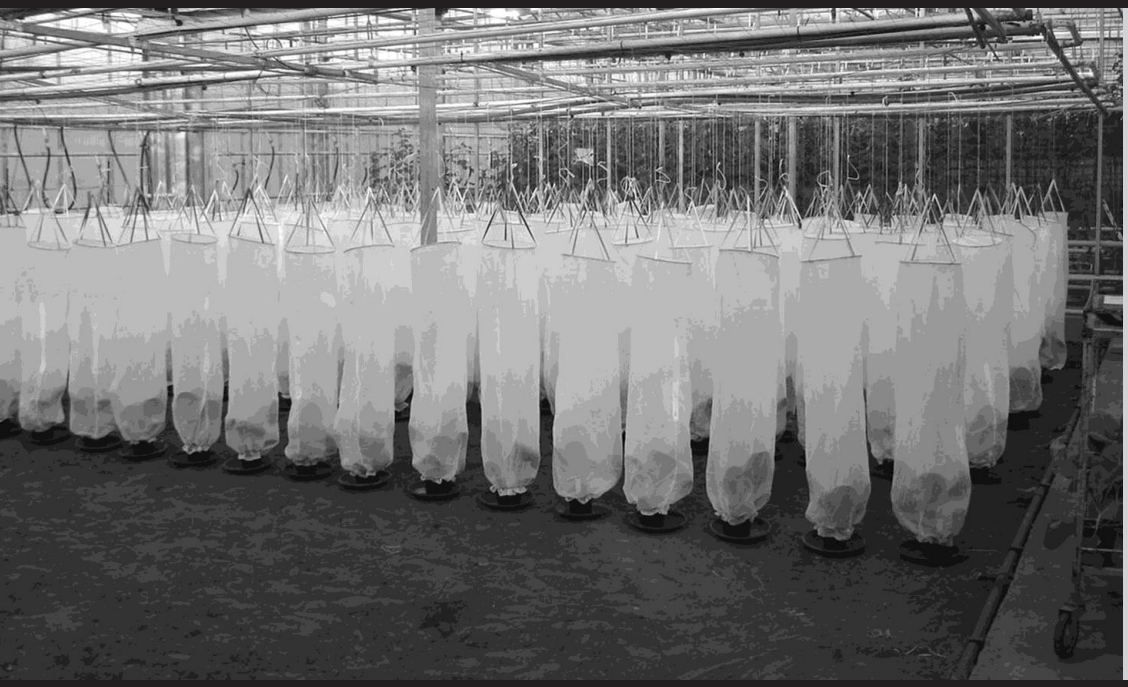

Chapter 6

Picture: Erik Poelman

Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field

Erik H. Poelman, Colette Broekgaarden, Joop J.A. van Loon & Marcel Dicke

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Abstract

Induction of plant defences by early-season herbivores can mediate interspecific herbivore competition. We have investigated plant-mediated competition between three herbivorous insects through studies at different levels of biological integration. We have addressed (a) gene expression, (b) insect behaviour and performance under laboratory conditions, and (c) population dynamics under field conditions. We studied the expression of genes encoding a trypsin inhibitor and genes that are involved in glucosinolate biosynthesis in response to early-season herbivory by *Pieris rapae* caterpillars in *Brassica oleracea* plants. Furthermore, we studied the interaction of these transcriptional responses with responses to secondary herbivory by the two specialist herbivores *P. rapae* and *Plutella xylostella,* and the generalist *Mamestra brassicae*.

***Pieris rapae*-induced responses strongly interacted with plant responses to secondary herbivory. Sequential feeding by specialist herbivores resulted in enhanced or similar expression levels of defence-related genes compared to primary herbivory by specialists. Secondary herbivory by the generalist *M. brassicae* resulted in lower gene expression levels than in response to primary herbivory by this generalist.**

Larval performance of both specialist and generalist herbivores was negatively affected by *P. rapae*-induced plant responses. However, in the field the specialist *P. xylostella* was more abundant on *P. rapae*-induced plants and preferred these plants over undamaged plants in oviposition experiments. In contrast, the generalist *M. brassicae* was more abundant on control plants and preferred undamaged plants for oviposition. *Pieris rapae* did not discriminate between plants damaged by conspecifics or undamaged plants. Our study shows that early-season herbivory differentially affects transcriptional responses involved in plant defence to secondary herbivores and their population development dependent upon their degree of host plant specialization.

Key words: induced defence, herbivore competition, sequential attack, *Pieris rapae, Plutella xylostella, Mamestra brassicae,* trypsin and protease inhibitor, glucosinolates, *LOX2*

Introduction

Interspecific competition between phytophagous insects is profoundly affected by indirect competition mediated by plant responses to attackers (Faeth 1986; Denno et al. 1995; Schoonhoven et al. 2005; Kaplan and Denno 2007). Although separated in time, an early-season herbivore may affect the performance of subsequent colonizers by altering plant nutritional, allelochemical or morphological quality (Agrawal 2000; Traw and Dawson 2002; Inbar and Gerling 2008). These herbivory-induced plant responses (Karban and Baldwin 1997) may alter the attractiveness of plant phenotypes to late-season herbivores and affect host-plant acceptance by these species (Shiojiri et al. 2002; Kessler et al. 2004; Rodriguez-Saona et al. 2005). Similarly, natural enemies such as parasitoid or predatory arthropods may be differentially attracted by plants that undergo single, simultaneous or sequential herbivore attack (Shiojiri et al. 2001; Moayeri et al. 2007; de Boer et al. 2008) and, thus, induced responses may affect the risk of predation for late-season herbivores (Shiojiri et al. 2001; Kaplan et al. 2007). Thereby, early-season herbivory may have long-lasting effects on the population structure of subsequent colonizers (Thaler et al. 1999). Plant-mediated interspecific competition has been identified for interactions between herbivores of the same feeding guild for both leaf chewers (Faeth 1986) and sap-feeders (Denno et al. 2000) as well as across feeding guilds (Kessler and Baldwin 2004; Viswanathan et al. 2005). Negative effects that species may have on each other are highly asymmetric (Inbar et al. 1999; Agrawal 2000; Denno et al. 2000) and, although less often reported, herbivory by one species may facilitate herbivory by other species (Martinsen et al. 1998; Kaplan and Denno 2007).

Herbivores may affect each other asymmetrically either when they differ in susceptibility to induced responses of the plant or when herbivory alters the plant quality differentially for the type of attacker (Inbar et al. 1999). Members of different attacker guilds have been found to elicit profound differences in plant transcriptional responses (de Vos et al. 2005). Piercing-sucking insects were found to affect the expression of salicylic acid (SA)-regulated genes, whereas leaf chewing insects affect the expression of genes regulated by the jasmonic acid pathway (JA) (Heidel and Baldwin 2004; de Vos et al. 2005; Zarate et al. 2007). Herbivory by members of the same feeding guild may elicit similar responses in plants (Reymond et al. 2004), but are often found to at least partly elicit different responses (Voelckel and Baldwin 2004a). These differences in elicited responses result from the damage signature of herbivores comprised of damage pattern and composition of biochemical components of their saliva (De Moraes et al. 1998; Dicke 1999; Roda et al. 2004; de Vos et al. 2005). SA- and JA pathways have been found to act antagonistically by inhibiting the response to a subsequent attacker of a different species (Thaler et al. 1999, 2002). Upon sequential attack the transcriptional response of a plant and its defensive products may be enhanced or redirected towards defence against the new attacker (Voelckel and Baldwin 2004b; Viswanathan et al. 2007). Thereby, defence induction by a first colonizer may interact with plant responses towards subsequent colonizers. Responses of individual genes to sequential herbivory are, however, understudied and have not been linked with their consequences for behaviour and population development of herbivores with different susceptibilities to direct plant defences. We here attend to plant-mediated competition by herbivorous insects through an approach addressing different levels of biological organisation.

Here, we study the response of four genes that are involved in direct defence of *Brassica oleracea* plants. These genes were selected based on previous studies, including a full transcriptomic analysis using a microarray, in which among 21 defence related genes the four selected genes were found to be induced in two cultivars of *B. oleracea* after her-

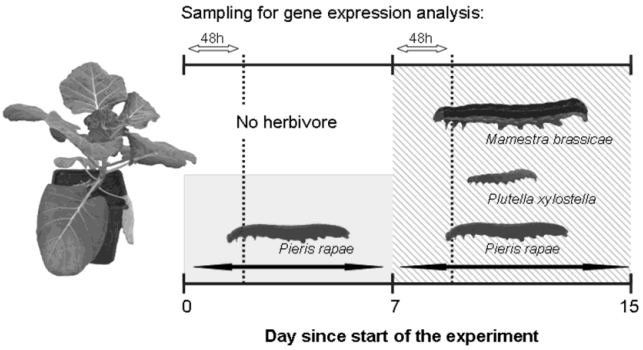

Figure 1. Experimental setup of the herbivore performance experiment. Half of the plants were exposed to *Pieris rapae* herbivory for 7 days; the other half served as undamaged control. Caterpillars of *P. rapae* were removed after 7 days. Afterwards, induced and control plants were infested with caterpillars of one of the three subsequent herbivores. These caterpillars fed for 8 days on the plant and were subsequently removed to assess their mass. Plant material to measure gene expression was collected after 48 hours of feeding by the first and second herbivore, time points are indicated by a stippled line. The experimental design was applied to two different cultivars of *B. oleracea*.

bivory by caterpillars of the Small Cabbage White *Pieris rapae* (Pieridae) (Broekgaarden et al. 2007; Zheng et al. 2007). The four genes were further selected based on their role in transcript of different types of direct defences against caterpillars. Two of the selected genes encode a trypsin inhibitor (*TI*) and a trypsin and protease inhibitor (*TPI*). The other two genes are *LIPOXYGENASE 2* (*LOX2*), playing a key role in JA biosynthesis, and *CYP83B1* that encodes a P450 enzyme involved in the biosynthesis of glucosinolates. These secondary metabolites are characteristic for Brassicaceae and are well-known to reduce the growth of insect herbivores (Giamoustaris and Mithen 1995; Fahey et al. 2001; Traw and Dawson 2002). We quantify the expression of the selected genes after herbivory by caterpillars of *P. rapae* that are the first caterpillars colonizing *Brassica* plants in the field in the Netherlands (Poelman unpublished data). We study the effect of early-season *P. rapae*-induced plant responses on plant gene expression, caterpillar performance, oviposition preference and population development of subsequently colonizing herbivores that differ in the degree of host plant specialization: the generalist *Mamestra brassicae* (Noctuidae) and the specialists *Plutella xylostella* (Yponomeutidae) and *P. rapae*. Effects are studied in two cultivars of *B. oleracea* that were found to differ most widely in resistance to herbivores among eight cultivars of *B. oleracea* (Poelman et al. 2008c).

We tested the hypothesis that an early-season herbivore differentially affects population structure of subsequent herbivores dependent upon their degree of host plant specialization (van der Meijden 1996; Agrawal 1999; Stotz et al. 2000). Generalist herbivores that accept a broad range of plants from different families as their host are typically affected by defences specific for a host-plant family. Specialists that feed on a narrow range of host plants, often belonging to a single family, have better adapted to specific secondary metabolites and often use these compounds to recognize their host plant (Jaenike 1990). Thus, induced plant responses may result in negative effects on population size of generalist herbivores, while no effects or even positive effects on abundance of specialist attackers may be predicted. We discuss the relationship between plant gene expression and observed behavioural responses of herbivores in the context of herbivore competition in the field, as mediated by induced plant defence.

Material and Methods

Plants and insects

To study plant-mediated herbivore competition, we used two cultivars of white cabbage *Brassica oleracea* var. *alba* L. that differ in resistance against herbivores: the susceptible

cultivar Badger Shipper (Centre for Genetic Resources, CGN-Wageningen, The Netherlands), and more resistant cultivar Rivera (Bejo Zaden BV, Warmenhuizen, The Netherlands) (Poelman et al. 2008c). Seeds of the cultivars germinated on peat soil (Lentse potgrond, No. 4) and two-week-old seedlings were transferred to 1.45-L pots containing the same potting soil. Pots were placed in a greenhouse, providing the plants with a 16/8 (day/night) photoperiod of SON-T light (500 μmol m^2/sec), 18ºC–26ºC and 40-70% relative humidity. When the plants were four weeks old, they were fertilised weekly by applying 100ml of the nutrient solution Kristalon Blauw (N-P-K) 19-6-20-3 micro (2.5 mg/l) to the soil. We used seven-week-old plants (having 12 leaves on average) in oviposition experiments and larval performance experiments. In these experiments, the larvae and adults of the Small Cabbage White *Pieris rapae* L. (Pieridae), the Diamondback moth *Plutella xylostella* L. (Yponomeutidae) and the Cabbage moth *Mamestra brassicae* L. (Noctuidae) originated from stock rearings of the Laboratory of Entomology, Wageningen University. They are maintained on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* L. cultivar Cyrus) in climatized rooms at 20-22ºC, 50-70% relative humidity and a 16/8 hour light/dark photoperiod. *M. brassicae* moths are offered only filter paper as oviposition substrate, without contact to cabbage plants.

Herbivore performance and sampling for gene expression analysis

To assess the effect of *P. rapae* feeding on gene expression and the performance of the subsequently feeding herbivores *P. rapae*, *P. xylostella* and *M. brassicae*, a greenhouse experiment was conducted (Figure 1). Ninety plants of the *B. oleracea* cultivars Badger Shipper and Rivera were placed in a greenhouse compartment with 16 h day and 8 h night period (22 ± 4 ºC, 60-70 % relative humidity). We infested half of the plants (45 per cultivar) with ten neonate *P. rapae* caterpillars per plant. The caterpillars were released on the youngest fully expanded leaf in a clip-cage (diameter 2.3 cm) to secure local damage and the plants were individually covered with nets to prevent the caterpillars from escaping. Empty cages were clipped onto control plants. After 48 hours of caterpillar feeding, we used a cork borer (2.3 cm diameter) to collect a leaf disk from the leaf containing the clip cage for each of the plants and removed the clip cages. Leaf disks of five individual plants per treatment were pooled and immediately frozen in liquid nitrogen, and stored at -80 ^{o}C until quantitative RT-PCR analysis of defence-related genes. After seven days of *P. rapae* herbivory, all caterpillars were removed and weighed. On the same day each of the four groups of 45 plants was divided into three groups of 15 plants, which were infested with ten neonate caterpillars of a second herbivore, i.e. either *P. rapae*, *P. xylostella* or *M. brassicae*. The clip cages containing the second herbivore were attached to the same leaf that was exposed to primary herbivory. Again, after 48 hours leaf material was collected as described above and clip cages were removed. When the second herbivore had been feeding for six more days without being caged (eight days feeding in total), caterpillars were removed and their mass was assessed.

RNA extraction and quantitative RT-PCR

As indicators of induced resistance we studied the expression of four genes involved in direct defence and their expression patterns under sequential herbivory. Gene-specific primers were designed for the four *B. oleracea* genes that have been identified to regulate direct defence pathways and to be significantly upregulated after *P. rapae* herbivory (Broekgaarden et al. 2007). The corresponding AGI codes of the *Arabidopsis thaliana* homologs and primer sequences are At1g47540 (*TRYPSIN INHIBITOR, TI*), LEFT 5'-CTG AAA GAA TAC GGA GGC AAC-3', RIGHT 5'-AAT ACC GCC ACT TAG AAT CTG

G-3'; At1g72290 (*TRYPSIN AND PROTEASE INHIBITOR, TPI*), LEFT 5'-TGG TGA CAA GTA GCT GTG GTG-3', RIGHT 5'-TCC AAG TTA TGG GCA GTG G-3'; At3g45140 (*LIPOXYGENASE 2, LOX2*), LEFT 5'-CTT TGC TCA CAT ACG GTA GAA GC-3', RIGHT 5'-CCT TTG CAT TGG GCT AGT TC-3'; At4g31500 (*CYTOCHROME P450 83B1, CYP83B1*), LEFT 5'-CCG GAA TAT CAT AGC CAC CTA TC-3', RIGHT 5'-CCT GAA GCA ATG AAG AAA GCT C-3'. Each of the four *Brassica oleracea* primers is above 88% homologous to the sequence of *Arabidopsis* primers. From the greenhouse experiment we obtained three biological replicas per time point and treatment, each consisting of a pool of five plants. Total RNA was isolated by using TRIzol reagent (Invitrogen) and purified using the RNeasy MinElute kit (Qiagen). According to the manufacturer's instructions, one µg of total RNA was treated with DNaseI (Invitrogen) and DNA-free total RNA was converted into cDNA using the iScript cDNA synthesis kit (Bio-Rad, Veenendaal, The Netherlands). Efficiency of cDNA synthesis was assessed by qRT-PCR using primers of the constitutively expressed gene *GAPDH* (*GAPDH*-LEFT; 5'-AGA GCC GCT TCC TTC AAC ATC ATT-3'; *GAPDH*-RIGHT; 5'-TGG GCA CAC GGA AGG ACA TAC C-3'). Quantitative RT-PCR analysis was done in optical 96-well plates with a MyiQ Single-Colour Real-Time PCR Detection System (Bio-Rad), using SYBR Green to monitor dsDNA synthesis. Each reaction contained 10 µl 2x IQ SYBR Green Supermix reagent (Bio-Rad), 10 ng cDNA, and 300 nM of each gene-specific primer in a final volume of 20 µl. All qRT-PCR reactions were performed in duplicate. The following PCR program was used for all PCR reactions: 95 ^{o}C for 3 min; 40 cycles of 95 ^{o}C for 30 sec and 60 ^{o}C for 45 sec. C_T (threshold cycle) values were calculated using Optical System Software, version 2.0 for MyIQ (Bio-Rad). Subsequently, C_T values were normalized for differences in cDNA synthesis by subtracting the C_T value of *GAPDH* from the C_T value of the gene of interest. Normalized gene expression was obtained from the equation $2^{-\Delta CT}$. All primers were tested for gene specificity by performing melt curve analysis and PCR products were sequenced.

Oviposition preference

To assess whether herbivory by *P. rapae* affects oviposition preferences of subsequently colonizing herbivores, we conducted oviposition preference experiments for each of the three herbivore species. Freshly eclosed adults of *P. rapae*, *P. xylostella* and *M. brassicae* were collected from the stock rearing, placed in separate cages where they were provided with a 10% sucrose solution and allowed to mate. After two days, pairs of each species were transferred to the experimental setup containing one of two choice situations. Preference tests, in which we offered an excised undamaged control leaf versus an excised leaf that was damaged by caterpillars of *P. rapae*, were carried out for the two *B. oleracea* cultivars separately. Damaged leaves were excised from plants that were infested with ten neonate *P. rapae* caterpillars, which had been feeding on the plants for seven days in a glasshouse compartment (22 ± 4 ºC, 60-70 % relative humidity and a 16/8 hour light/dark photoperiod). At the start of the oviposition experiment caterpillars were removed from the leaves and the excised leaves were placed in glass vials containing tap water to keep the leaves turgid.

Oviposition experiments with *P. rapae* were conducted in oviposition cages (67 x 50 x 75 cm) placed in a glasshouse compartment that was kept at 22 ± 4 ºC and 50-70% relative humidity. During the experiment the oviposition cages were illuminated by SON-T, 500W sodium vapour lamps, in addition to natural daylight. Butterflies were allowed to oviposit on the leaves from 9:00 am to 2:00 pm. Afterwards, the leaves were removed from the cages and eggs were counted.

Preference tests on *M. brassicae* and *P. xylostella* were run in plastic cylinder-shaped cages (22 cm height, 13 cm diameter), which were placed in a climatized room (20 ± 2 ºC, 50-70% relative humidity and a 16/8 hour light/dark photoperiod). Moths were allowed to oviposit for 24 hours before the leaves were removed and the number of eggs was counted. For *M. brassicae* we additionally scored the number of egg batches on each of the leaves. Both species also laid eggs on the cage walls; these were counted but excluded from the preference analysis. Results from individuals that laid eggs only on the cage walls or no eggs at all were also excluded from the analysis. This resulted in exclusion of 15 out of the 92 tests for *P. xylostella* and 24 out of 96 tests for *M. brassicae*.

Common garden experiment

We conducted a common garden experiment to test whether early-season herbivory by *P. rapae* affects population development of subsequently colonizing herbivores in the field. In week 19 (11 May) in 2006, 40 plots (6 x 6 m), each containing a monoculture of 49 plants of one of the two cultivars were established in an agricultural field in the vicinity of Wageningen, The Netherlands, using a randomized design. Five-week-old plants were planted in a square of 7 x 7 plants with a spacing of 75 cm between plants. Plots were isolated by an area of 6 meters that was sown with a grass mixture of *Lolium* and *Poa* species. In Week 21 (22 May), all plants on half of the plots per cultivar (n=10) were infested with two second instar *P. rapae* caterpillars. The caterpillars were allowed to feed for seven days and thereafter removed from the plant. The plants were exposed to naturally occurring herbivore populations. From week 23 (5 June) until week 36 (8 September), the central 9 plants of each plot were surveyed weekly for the presence of *P. rapae*, *P. xylostella* and *M. brassicae* caterpillars. Each plant was surveyed by investigating both sides of all its leaves.

Statistical analysis

Herbivore performance, expressed as mass of eight-day-old caterpillars was analysed by ANOVA for the factors cultivar and induction treatment (control or induced).

Oviposition preference was analyzed by paired t-tests for *P. rapae* and *P. xylostella*. As the data on *M. brassicae* egg batches and eggs were not normally distributed, we applied a Wilcoxon matched-pairs signed ranks test.

We analysed the population development over time of *P. rapae*, *P. xylostella* and *M. brassicae* using a structured repeated measurements mixed model. The models were constructed with the Proc Mixed function of SAS 9.1, using a repeated structure type AR (1). The dependent variable, the average number of caterpillars per plant in a plot, was log normalized and modeled by the factors cultivar, week (23-36), treatment (control, induced) and the factorial interactions. Log-normalized gene expression was analysed using an unstructured repeated measurements mixed model for the RNA pools of repeatedly sampled plants (Proc Mixed function of SAS 9.1). Expression for each of the four genes was modeled for the factors cultivar, pre-treatment (control or *P. rapae* induced), herbivore (*P. rapae*, *P. xylostella* or *M. brassicae*), time and the factorial interactions.

Results

Gene expression

Fourty eight hours after introducing *P. rapae* as a first herbivore the four selected genes all showed an induced expression compared to control plants in both cultivars (Table 1A, Figure 2 Week 1). After one week of primary herbivory by *P. rapae*, the caterpillars were removed from the plants and in week two a second herbivore was introduced to both con-

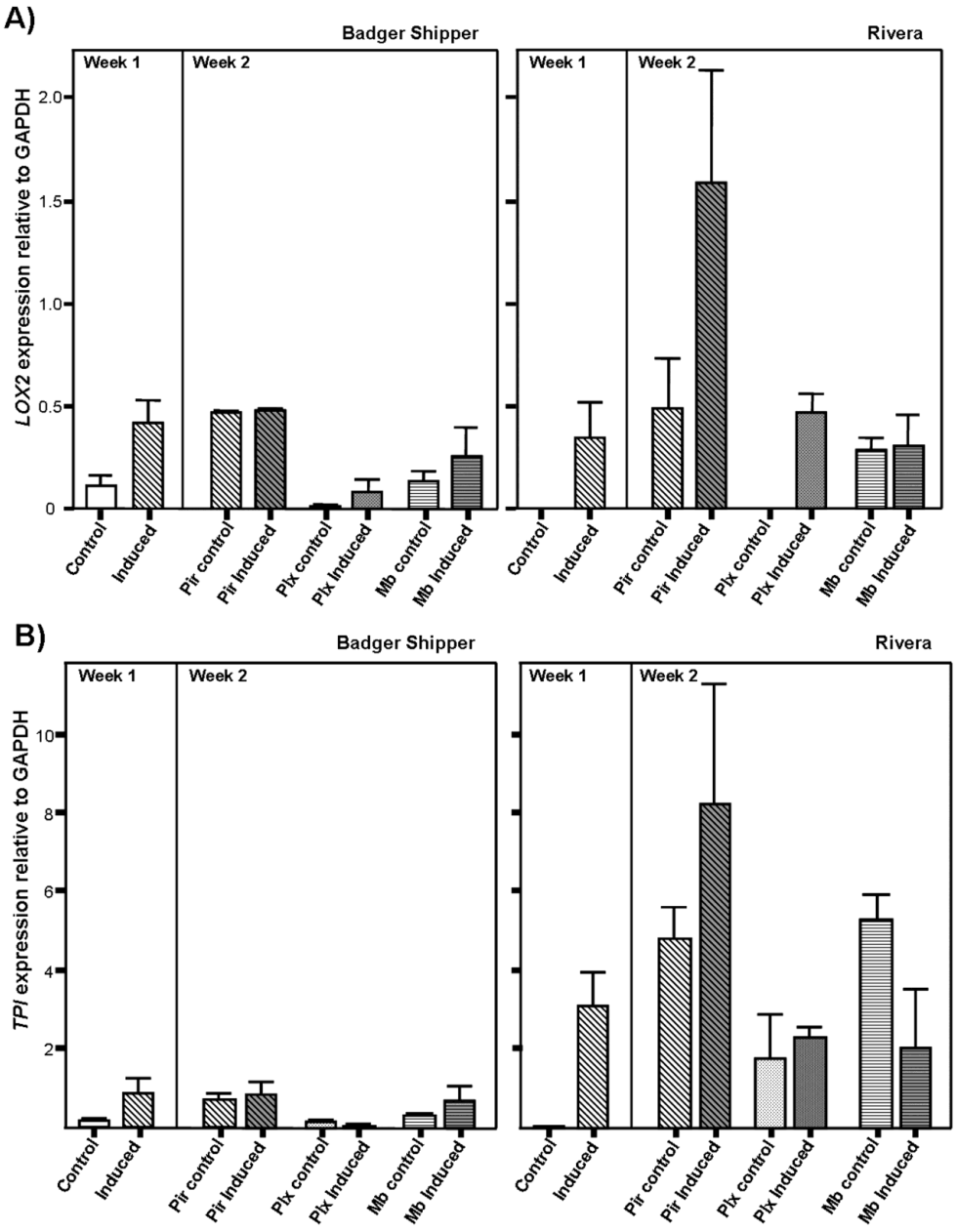

Figure 2. Expression (mean ± SE) of four direct defence related genes *LOX2* (A), *TPI* (B), *CYP83B1* (C), and *TI* (D) in two *B. oleracea* cultivars. The two bars in the left panel of each graph per cultivar shows gene expression in control plants and in plants exposed to 48h of *P. rapae* feeding (induction treatment) in week 1, the right panel shows gene expression 48h after subsequent herbivory by *P. rapae* ((Pir) diagonally striped), *P. xylostella* ((Plx) spotted), *M. brassicae* ((Mb) striped) in week 2. Expression without herbivore induction is presented in white bars, single herbivore treatment in light grey and subsequent herbivory in dark grey. Transcript levels in Rivera are higher than in Badger Shipper and differential for herbivore species; the responses to secondary herbivory significantly interact with the responses to herbivory by the first attacker (see Table 1B).

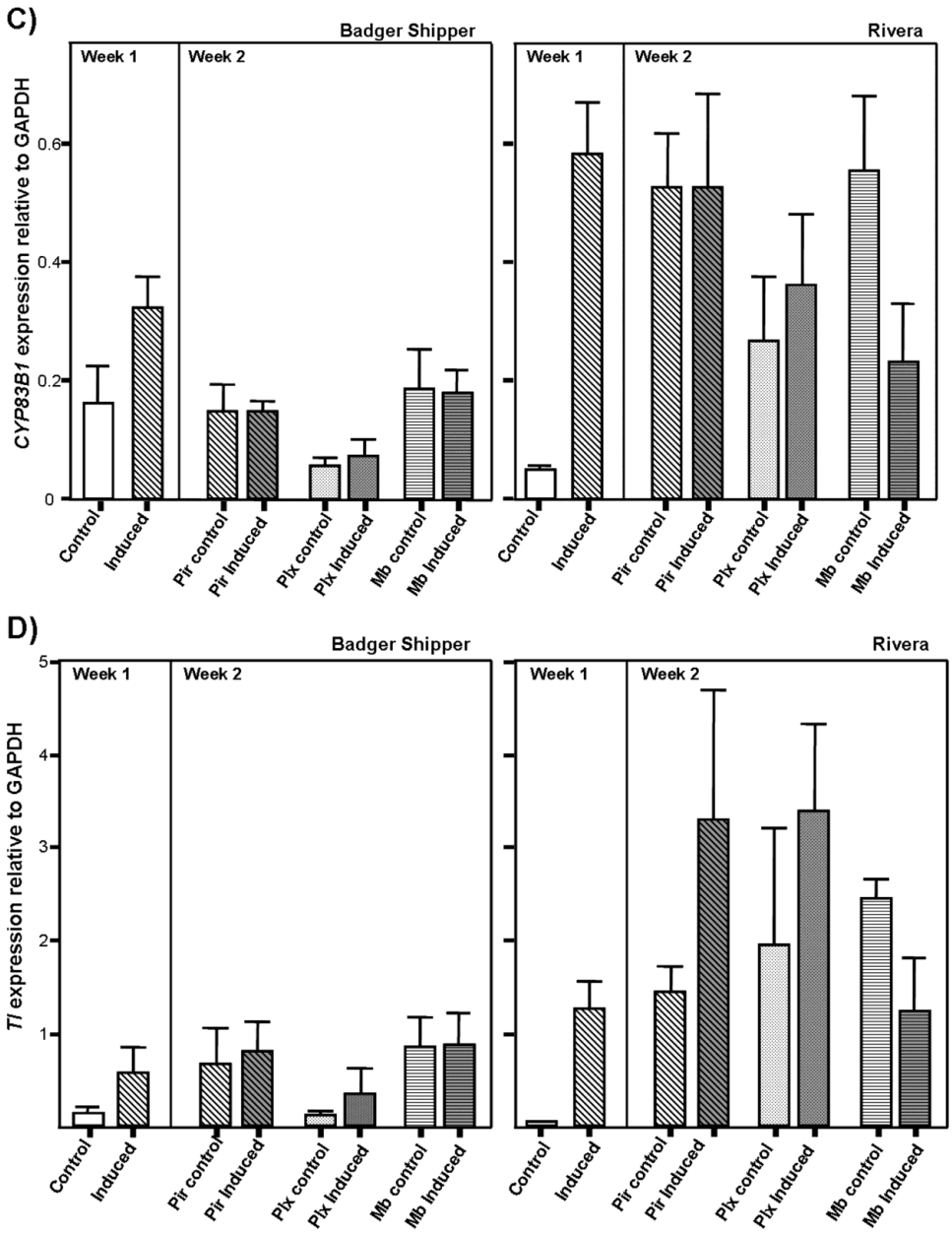
C)
Badger Shipper
Rivera
Week 1
Week 2
CYP83B1 expression relative to GAPDH
0
0.2
0.4
0.6
Control
Induced
Pir control
Pir induced
Plx control
Plx induced
Mb control
Mb induced
D)
Tl expression relative to GAPDH
1
2
3
4
5

Table 1. *F*-test for the repeated measurement Mixed model analysis of the expression of four direct-defence-related genes, testing whether gene expression levels are affected by the factors cultivar, caterpillar species feeding on the plant, the *P. rapae* induction treatment and the time of measurement since herbivore feeding (A), as well as all factorial interactions (B). For each factor we provide F-statistics and the corresponding P-value. Boldface type indicates significant terms (α = 0.05).

A)	Factor							
	Cultivar (1) d.f. = 1		Treatment (2) d.f. = 1		Herbivore (3) d.f. = 2		Time (4) d.f. = 1	
Gene	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
LIPOXYGENASE 2 (*LOX2*)	0.32	0.576	81.58	**<0.001**	24.42	**<0.001**	84.29	**<0.001**
TRYPSIN INHIBITOR (*TI*)	22.79	**<0.001**	88.71	**<0.001**	12.49	**<0.001**	169.74	**<0.001**
TRYPSIN, PROTEASE INHIBITOR (*TPI*)	20.05	**<0.001**	32.75	**<0.001**	4.86	**0.017**	68.28	**<0.001**
CYTOCHROME P450 83B1 (*CYP83BI*)	19.36	**<0.001**	43.03	**<0.001**	9.92	**<0.001**	6.64	**0.017**

B)	Interaction																			
	(1 * 2) d.f. = 1		(1 * 3) d.f. = 2		(1 * 4) d.f. = 1		(2 * 3) d.f. = 2		(2 * 4) d.f. = 2		(3 * 4) d.f. = 1		(1 * 2 * 3) d.f. = 2		(2 * 3 * 4) d.f. = 2		(1 * 3 * 4) d.f. = 1		(1 * 2 * 3 * 4) d.f. = 4	
Gene	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
LOX2	3.33	0.081	2.43	0.109	21.87	**<0.001**	2.16	0.138	54.69	**<0.001**	13.53	**<0.001**	4.50	**0.022**	7.91	**0.002**	0.02	0.889	6.03	**0.002**
TI	1.02	0.323	4.72	**0.019**	20.26	**<0.001**	1.89	0.174	219.02	**<0.001**	10.82	**<0.001**	6.59	**0.005**	11.22	**<0.001**	7.93	**0.010**	9.19	**<0.001**
TPI	0.30	0.588	3.10	0.064	3.00	0.096	0.46	0.640	38.75	**<0.001**	4.52	**0.022**	2.96	0.071	3.77	**0.038**	0.79	0.382	4.03	**0.012**
CYP83BI	1.76	0.198	4.14	**0.029**	53.30	**<0.001**	0.61	0.550	109.17	**<0.001**	6.67	**0.005**	2.97	0.071	3.99	**0.032**	15.91	**0.001**	9.31	**0.001**

trol and pre-infested plants. After introducing *P. rapae* as a second herbivore on Rivera, pre-infested plants showed higher levels of expression for *LOX2, TPI* and *TI* compared to plants without pre-infestation (Table 1B, Figure 2A-B-D Week 2). In the more susceptible cultivar Badger Shipper, pre-infestation with *P. rapae* did not affect the expression of any of the selected genes when *P. rapae* was introduced as secondary herbivore. When *P. xylostella* was introduced as a second herbivore, only *LOX2* showed a higher level of expression in pre-infested plants of Rivera. Interestingly, in Rivera plants that were not pre-infested no *LOX2* expression was detected after 48 h of *P. xylostella* infestation. Expression of the other genes was unaffected by pre-infestation in both cultivars (Table 1B, Figure 2 Week 2). When *M. brassicae* was introduced as a second herbivore on Rivera *TPI*, *CYP83B1*, and *TI* showed lower levels of expression compared to plants without pre-infestation (Figure 2 B-C-D Week 2). None of the four genes showed differential expression after primary or secondary herbivory by *M. brassicae* in Badger Shipper. Herbivory by a first colonizing herbivore differentially affected the gene expression patterns in response to a second herbivore and the magnitude of expression differed between the cultivars (Table 1B, Figure 2 Week 2).

Herbivore performance

Corresponding with the induction of defence-related genes by *P. rapae*, growth of secondary herbivores was negatively affected by primary feeding of *P. rapae* (Table 2, Figure 3). Induced defences in cultivar Badger Shipper reduced the performance of all three secondary herbivores by about 30%. In cultivar Rivera induced defences reduced the performance of the generalist *M. brassicae* by 24% and the performance of the specialists by 18% (*P. rapae*) and 14% (*P. xylostella*). The *P. rapae* caterpillars that were used to induce plant defences had a lower mass after feeding on Rivera compared to Badger Shipper (ANOVA, $F_{1,672} = 49.67$, $P<0.001$), supporting earlier findings of lower herbivore performance on control plants of Rivera (Poelman et al. 2008c). Also the caterpillars used in week 2 that were feeding on control plants had a reduced growth on Rivera compared to Badger Shipper. However, the reduction was only 10% for the two specialists (*P. rapae* and *P. xylostella*) while it was 20% for the generalist *M. brassicae*. A significant effect of cultivar on caterpillar growth was only found for the generalist *M. brassicae* (Table 2, Figure 3). The two specialist herbivores were less affected by constitutive and induced foliar quality of the cultivars than the generalist.

Oviposition preference

Female butterflies of *P. rapae* did not discriminate between control leaves or leaves that were previously damaged by conspecific caterpillars; this was recorded in separate choice tests for each of the two cultivars (paired t-test, Badger Shipper: $t = 0.904$, d.f. = 24, $P = 0.375$; Rivera: $t = 0.013$, d.f. = 23, $P = 0.990$) (Figure 4). In similar tests, female moths of the specialist *P. xylostella* preferred leaves damaged by *P. rapae* caterpillars over control leaves (paired t-test, Badger Shipper: $t = -6.095$, d.f. = 36, $P <0.001$; Rivera: $t = -8.696$, d.f. = 39, $P < 0.001$). In contrast, *M. brassicae* moths laid more egg batches on control leaves than on leaves that had been damaged by *P. rapae* caterpillars (Wilcoxon matched-pair signed ranks test, Badger Shipper $N = 37$, $Z = -1.990$, $P = 0.047$, Rivera: $N = 33$, $Z = -3.465$, $P = 0.001$) (Figure 4), which was also reflected in the number of eggs deposited (Wilcoxon matched-pair signed ranks test, Badger Shipper $N = 37$, $Z = -2.384$, $P = 0.017$, Rivera: $N = 33$, $Z = -3.315$, $P = 0.001$). *P. xylostella* deposited 40% and *M. brassicae* 23% of their eggs on the cage walls.

Table 2. ANOVA for the effect of cultivar and *P. rapae* induction treatment on the performance of three herbivores. For each factor we provide F-statistics and the corresponding P-value. Boldface type indicates significant terms ($\alpha = 0.05$).

		Factor				Interaction	
		Cultivar (1) d.f. = 1		Treatment (2) d.f. = 1		(1 * 2) d.f. = 1	
Herbivore	n	*F*	*P*	*F*	*P*	*F*	*P*
Pieris rapae	503	0.97	0.325	54.94	**<0.001**	7.04	**0.008**
Plutella xylostella	342	0.77	0.381	35.13	**<0.001**	6.93	**0.009**
Mamestra brassicae	430	16.62	**<0.001**	21.81	**<0.001**	0.43	0.515

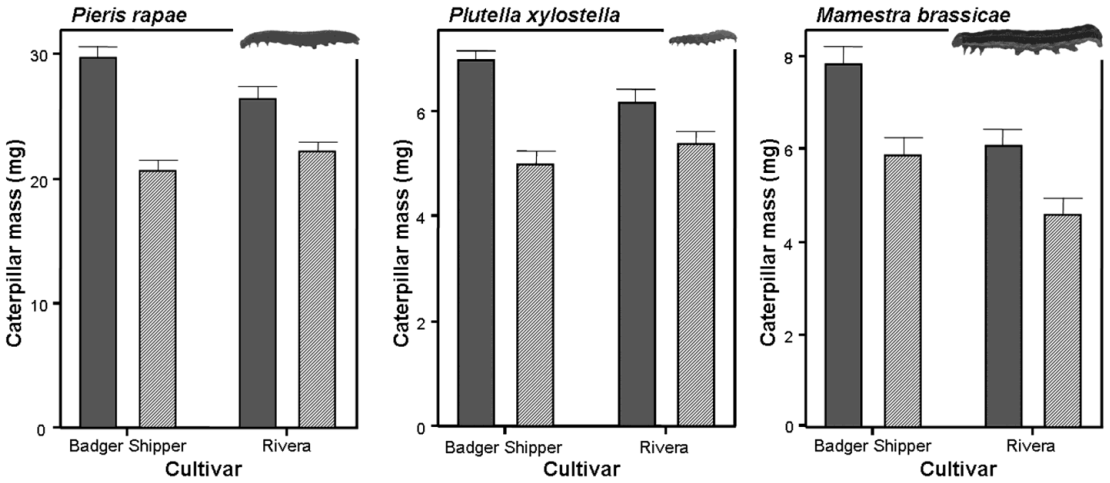

Figure 3. Performance of caterpillars of *P. rapae*, *P. xylostella* and *M. brassicae* on control (dark grey) and *P. rapae* induced plants (light grey) of two cultivars of *B. oleracea*. Bars represent average with SE. Caterpillars of all three species were smaller when feeding on plants that were previously damaged by *P. rapae* caterpillars. Feeding on control plants of Rivera resulted in smaller caterpillars for all three species compared to feeding on Badger Shipper. However, induction effect of *P. rapae* feeding on a subsequent specialist herbivore was less strong for Rivera compared to Badger Shipper. The induction effect had an equally strong effect on the generalist *M. brassicae* in both cultivars.

Common garden experiment

In the field all three herbivore species showed clear population fluctuations over time and had two or more generations (Table 3, Figure 5). In line with the oviposition preference experiment, *P. xylostella* was more abundant on induced plants, whereas *M. brassicae* was less abundant on induced plants that had received early-season herbivory by *P. rapae* caterpillars. *Plutella xylostella* and *M. brassicae* were more abundant on the more susceptible cultivar Badger Shipper. Population size of *P. rapae* itself was neither affected by cultivars nor by induction of defence by conspecific caterpillars (Table 3, Figure 5).

Discussion

Induced defences in plants resulting from early-season herbivory are known to differentially affect population structure of subsequent colonizers (Kaplan and Denno 2007). Here we experimentally demonstrated that the degree of host-plant specialization of secondary herbivores can explain differential outcomes of induced plant defences. Early-season her-

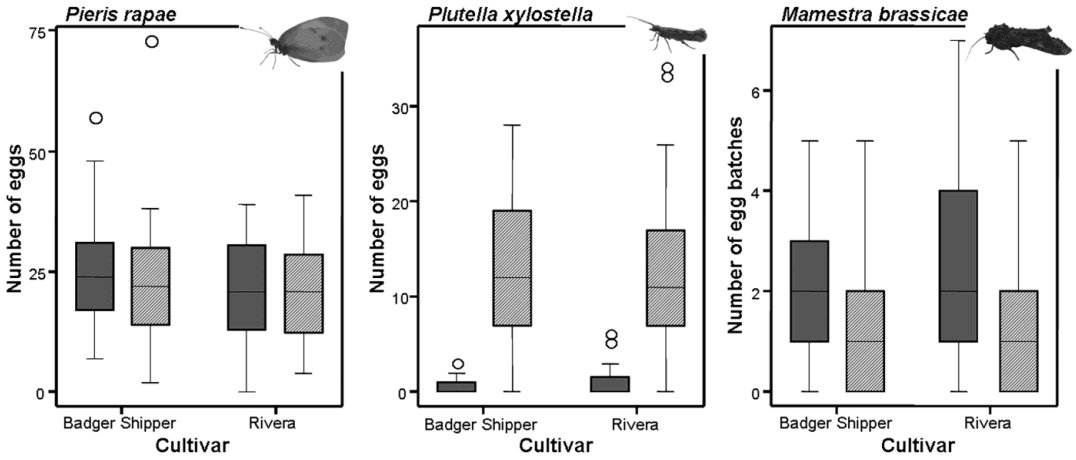

Figure 4. Oviposition of butterflies of *P. rapae*, *P. xylostella* and *M. brassicae* on excised leaves of control (dark grey) and *P. rapae* induced plants (light grey) tested for two cultivars of *B. oleracea*. The boxes represent the first to third quartile range with the median indicated by a line across the box. Outlyers are presented by open circles. The specialist *P. rapae* did not discriminate between damaged and control leaves, whereas the specialist *P. xylostella* preferred leaves that were damaged by *P. rapae*. The generalist *M. brassicae* oviposited more on control leaves.

bivory reduced the population size of the generalist *M. brassicae*, whereas it resulted in a larger population size of the specialist *P. xylostella*. These field results are in concordance with differences in oviposition preference as observed under laboratory conditions. Induced defence after *P. rapae* feeding, as represented by the upregulation of four defence-related genes, resulted in enhanced resistance to all three herbivore species (generalist and specialists) tested as subsequent colonizers in the greenhouse. Furthermore, gene transcript levels in response to the three secondary herbivores interacted differentially with gene induction due to early-season herbivory.

Gene expression after secondary herbivory

It is well known that insect herbivores differentially induce plant defences and that induced defences by an herbivore species may affect the performance of secondary herbivores differentially (Agrawal 1999; 2000; Stotz et al. 2000; Traw and Dawson 2002). *Pieris rapae* feeding on *Brassica oleracea* plants in our study induced the expression of *LOX2*, a gene that plays a key role in JA biosynthesis. The activation of *LOX2* likely results in enhanced JA levels, leading to the mobilization of an array of defences. Simultaneously, more specific direct defence-related genes such as the genes encoding trypsin inhibitor (*TI*) and trypsin and protease inhibitor (*TPI*) showed an induced expression after *P. rapae* feeding. *CYP83B1*, whose product has a crucial role in the production of indole glucosinolates (Bak et al. 2001; Hansen et al. 2001) was also induced by *P. rapae* feeding. Induction of *CYP83B1* by *P. rapae* is in concordance with our previous finding that *P. rapae* feeding on the cultivars elicits an increase in foliar indole glucosinolate concentration (Poelman et al. 2008). Proteins with trypsin inhibitor activity and glucosinolates are all known to reduce the performance of generalist and specialist herbivores (Ryan 1990; Broadway 1995; Fahey et al. 2001; Traw and Dawson 2002; Glawe et al. 2003; Telang et al. 2003), including the herbivores investigated here (Li et al. 2000; Agrawal and Kurasige 2003). The higher level of expression of these genes in Rivera compared to Badger Shipper corre-

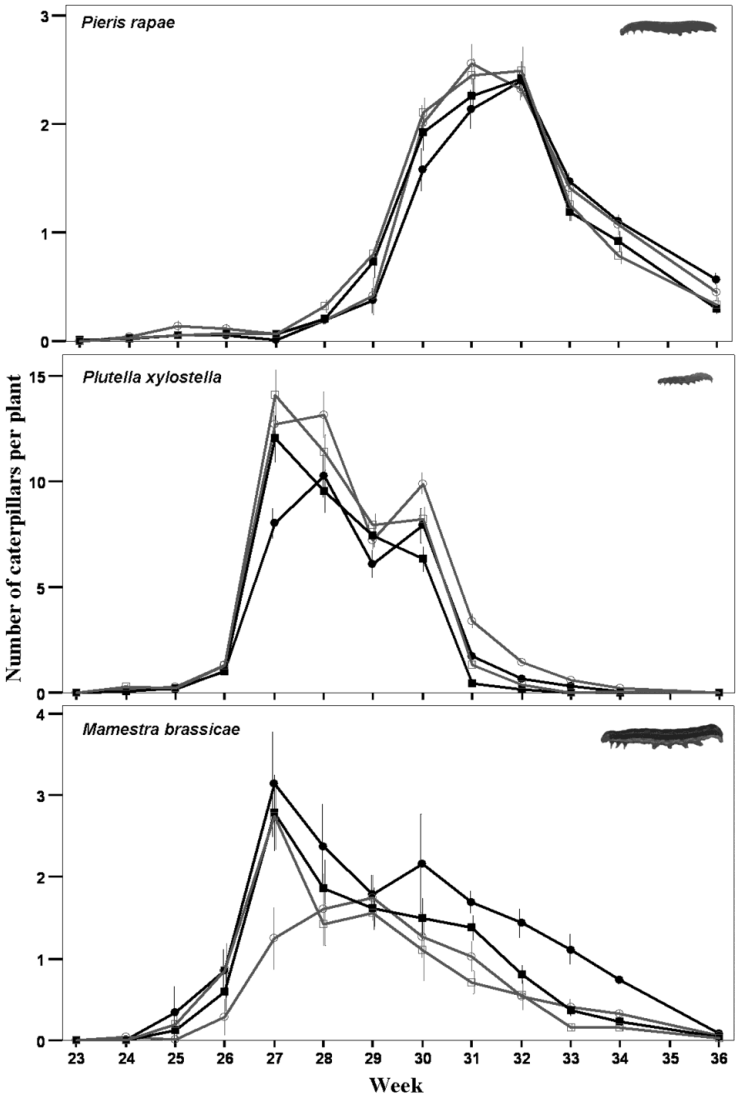

Figure 5. Population development of two specialist (*Pieris rapae* and *Plutella xylostella*) and a generalist herbivore (*Mamestra brassicae*) on *P. rapae*-damaged plants and control plants of two cultivars of *B. oleracea* in the field. The black lines with closed symbols represent control plants, the grey lines with open symbols plants that were damaged by *P. rapae* in the beginning of the field season. Circles represent cultivar Badger Shipper, squares represent cultivar Rivera. Population size of *P. rapae* was not affected by cultivar or treatment. The specialist *P. xylostella* had a higher population level on induced plants, whereas the generalist *M. brassicae* was less common on induced plants. Both herbivores were more abundant on Badger Shipper.

sponded with a poorer performance of herbivores on Rivera. *Pieris rapae* infestation induced the expression of all four of these genes and this corresponded with a poorer performance of the generalist and specialist herbivores than on plants without pre-infestation. Moreover, *P. rapae*-induced plant defences more strongly affected the growth of the generalist herbivore than that of the specialists. The induction of *CYP83B1* by *P. rapae* likely results in enhanced levels of glucosinolates (Poelman et al. 2008c) and may, therefore, account for the differential effect of induced defences on specialist and generalist herbivores. Specialists are found to be less sensitive to glucosinolates than generalists (Giamoustaris and Mithen 1995; van der Meijden 1996; Gols et al. 2008b).

The performance of secondary herbivores is, however, also dependent on the defence response elicited by these herbivores themselves. Plants that had been induced by initial herbivory may be physiologically constrained in responding to a subsequent herbivore by negative cross-talk between defence pathways (Thaler et al. 1999, 2002; Paul et al.

Table 3. *F*-test for repeated measurements Mixed model analysis of the factors cultivar and the treatment of early- season herbivory by *P. rapae* affecting herbivore population development in the field. For each factor we provide F-statistics and the corresponding P-value. Boldface type indicates significant terms ($\alpha = 0.05$).

	Factor						Interaction							
	Cultivar (1) d.f. = 1		Treatment (2) d.f. = 1		Time (3) d.f. = 12		(1 * 2) d.f. = 12		(1 * 3) d.f. = 12		(2 * 3) d.f. = 12		(1 * 2 * 3) d.f. = 12	
Herbivore	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*	*F*	*P*
P. rapae	0.02	0.886	2.15	0.152	243.63	**<0.001**	3.71	**<0.001**	0.06	0.806	0.61	0.838	0.73	0.721
P. xylostella	45.81	**<0.001**	36.29	**<0.001**	480.80	**<0.001**	13.76	**<0.001**	0.52	0.478	3.17	**<0.001**	0.68	0.767
M. brassicae	5.34	**0.027**	18.80	**<0.001**	75.54	**<0.001**	2.81	**0.001**	3.28	0.079	1.77	0.051	0.97	0.475

2000; Viswanathan et al. 2007; Zarate et al. 2007). This may result in canalization of defences by a first attacker, thereby limiting the response to a second herbivore (Viswanathan et al. 2007), or redirection of defences towards the second herbivore and away from those elicited by primary herbivory (Voelckel and Baldwin 2004b) or enhanced defence against a second attacker (de Vos et al. 2006). Here, we found that the three herbivores differentially induced plant defence-related genes (*LOX2*, *TI*, *TPI* and *CYP83B1*) when they infested the plant as primary herbivore (i.e. feeding in week 2 on previously undamaged control plants). However, the responses to secondary herbivory by these herbivores interacted with the response to initial herbivory. When *P. rapae* caterpillars induced plants that were previously damaged by conspecific caterpillars, the expression of *LOX2*, *TPI* and *TI* was higher than in plants without pre-infestation. Similarly, expression of *LOX2* was higher under secondary herbivory by *P. xylostella* compared to primary herbivory by this specialist herbivore. Interestingly, *LOX2* was not expressed when *P. xylostella* attacked plants as primary herbivore. This indicates that *P. xylostella* may be able to suppress plant responses, but this suppression was abolished when plants had already responded to previous damage by *P. rapae*. The other three genes did not show significant differences in expression levels by sequential herbivory of *P. xylostella* on plants previously infested with *P. rapae* versus control plants. Thus, sequential herbivory by the same herbivore or by two specialist herbivores resulted in similar or even enhanced response of plants as expressed in the four defence-related genes investigated here. However, when the generalist *M. brassicae* was introduced as secondary herbivore, the *TI* and *TPI* genes, encoding for proteins with trypsin inhibitor activity and a gene involved in glucosinolate biosynthesis (*CYP83B1*) were expressed at a lower level than under primary herbivory by *M. brassicae*. This may indicate that plants redirected their defence response to the secondary herbivore by spending resources on other defence-related genes that were not measured. Canalization or redirection of defences by a plant may depend on the similarity or dissimilarity respectively between the degrees of host plant specialization of sequentially attacking herbivores, adding an important factor to plant defence under multiple herbivore attack.

Preference and performance of herbivores

The effect of induced defences on herbivore competition is often valued in terms of herbivore performance. Only negative effects of induced defence on herbivore performance have been identified, including our results presented here. If there is selection on host plant acceptance and we assume that females prefer resources with maximal offspring performance (Thompson 1988; Mayhew 1997), females of any species are selected to avoid induced plants. Many herbivore species indeed avoid plants that are induced by conspecifics (Sato et al. 1999; De Moraes et al. 2001), which provides merit for relying on herbivore performance reduction only in quantification of induced defence. However, in brassica-

ceous plants glucosinolates and their breakdown products, isothiocyanates, typically stimulate host plant acceptance by specialists and deter generalists (Reed et al. 1989; van Loon et al. 1992; Renwick et al. 1992, 2006; Riggin-Bucci and Gould 1996). Although some literature contradicts with this generalization (Rojas 1999, Sato et al. 1999), induction of these defences may thus promote the presence of specialist herbivores. Their adaptation to specific chemical defences characteristic for a host plant family co-occurred with selection on host plant recognition based on the presence of these compounds (Wheat et al. 2007). However, specialist preference for induced compared to undamaged plants may depend on the plant species tested. Lu et al. (2004) showed that *B. oleracea* plants induced with the chemical elicitor JA were more attractive to *P. xylostella* moths than control plants, but the same treatment in the congeneric species *B. campestris* lead to avoidance of induced plants by moths. The absence of preference behaviour in *P. rapae* and the preference for induced plants by *P. xylostella* found in this study means that these specialists selected host plants that are nutritionally suboptimal for performance of their offspring. Suboptimal nutrition may, however, be traded off against ecological advantages arising from co-occurring with other herbivores. The specialist *P. xylostella* is known to prefer damaged plants, including those damaged by *P. rapae* (Shiojiri et al. 2002). The presence of *P. rapae* was found to create an enemy-free space for *P. xylostella* by decreasing the searching efficiency of specialist parasitoid wasps (Shiojiri et al. 2001, 2002). Species that occur later in the season, such as *P. xylostella* in The Netherlands, will always be confronted with a diversity of induced plant phenotypes caused by previous colonizers on top of abiotically extended plant phenotypes. Selection on resource choice has likely been driven by the balance between costs and benefits of accepting plants with different herbivore communities and associated induced defences. Our results show that differences in host plant acceptance mechanisms of generalist and specialist herbivores result in contrasting outcomes of induced plant defences in terms of herbivore abundance.

Community effect of induced defence

The differential effect of *P. rapae*-induced defences on host plant preference of generalist and specialist herbivores extended to long lasting effects on their population structure in the field. In agreement with the results from the laboratory studies, the generalist *M. brassicae* was indeed more common on control plants, whereas *P. xylostella* was more abundant on plants that had experienced early-season herbivory by *P. rapae*. In general, induced plants are found to harbour lower numbers of herbivores (Faeth 1986; Long et al. 2007), which applies to both leaf chewers and sap feeding herbivores (Denno et al. 2000; Viswanathan et al. 2005). However, induced responses of plants negatively affect the population structure of generalist herbivores in particular (Giamoustaris and Mithen 1995; Long et al. 2007), which supports our finding of *M. brassicae* avoiding induced plants. After silencing of the jasmonate cascade in which *LOX* is a key gene, Kessler et al. (2004) recorded for wild tobacco plants that the abundance of generalist herbivores is promoted by the lack of JA-dependent induced plant defences. These JA-silenced plants even became susceptible to generalists that are absent on control plants in wild tobacco (Kessler et al. 2004). Specialist herbivores were found to respond less profoundly to induced defences, but only few studies have found larger numbers of individuals on induced plants (Kaplan and Denno 2007). All studies that show an increase in herbivore numbers, including our results presented here, found that this relates to specialists (Giamoustaris and Mithen 1995; Martinsen et al. 1998; van Zandt and Agrawal 2004). Our results thus support the increasing evidence that induced defences are resulting in asymmetric competition between generalist and specialist herbivores in the field.

In conclusion, we have addressed plant-mediated competition between herbivores at different levels of biological integration. Our results show that a primary early-season colonizing herbivore has differential long lasting effects on the population size of subsequently colonizing herbivore species. These subsequent colonizers differentially induce gene expression in the plant in another way than when they would have been primary colonizer themselves. This suggests that the order of herbivore attack mediates differentiation of plant defence phenotypes and that these in their turn will affect community structure of herbivores (van Zandt and Agrawal 2004). As a result, generalist herbivores may be less abundant on plants that were previously damaged, whereas specialists may become more abundant on induced plants. Induced defences of the plant therefore not only shape herbivore abundance but may also diversify the herbivore community to become dominated by specialists.

Acknowledgements
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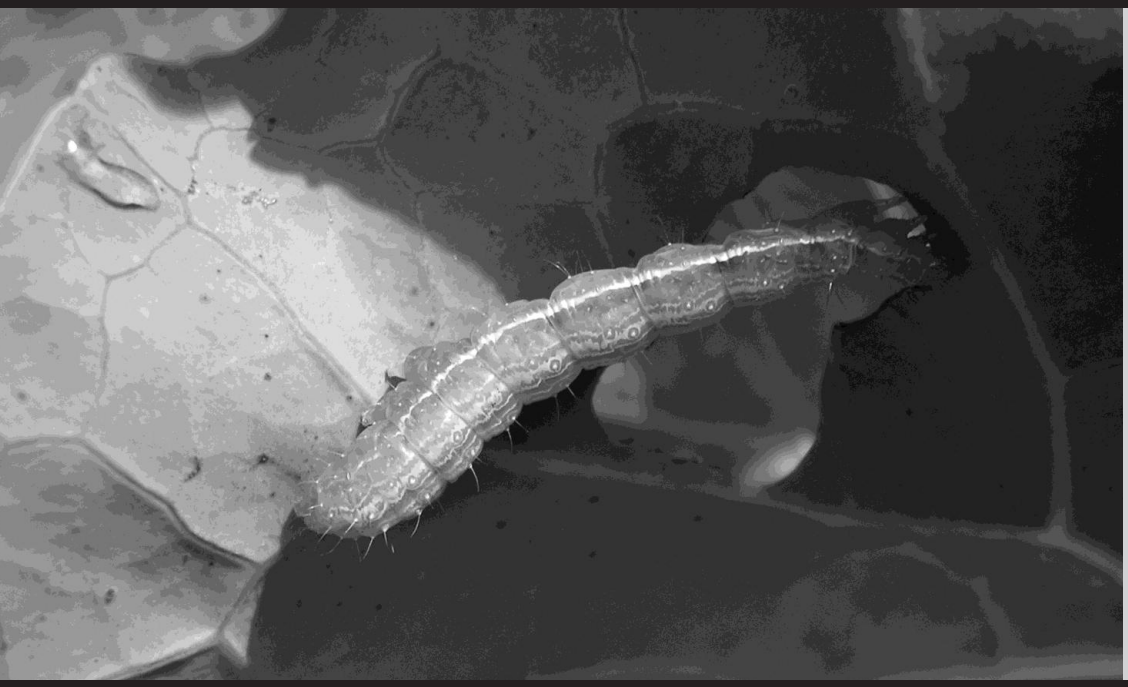

Chapter 7

Picture: Erik Poelman

Community-wide effects of induced plant responses: trade-offs between induced resistance to generalist and susceptibility to specialist herbivores

Erik H. Poelman, Joop J.A. van Loon, Nicole M. van Dam, Louise E.M. Vet & Marcel Dicke

Submitted

Abstract

Here we experimentally show that early-season herbivory by caterpillars of *Pieris rapae* induces responses in *Brassica oleracea* plants that affect the diversity of the herbivorous insect community associated with the plant later in the season. Generalist herbivores avoided plants with induced defences whereas these plants were colonized preferentially by specialists. The specialists were members of different feeding guilds, which demands a defence response of the plants at multiple fronts. Induced plant responses to an early-season herbivore thereby not only resulted in a different herbivore community composition, but also led to induced susceptibility of plants to a wide range of specialist herbivores. Our results show that early-season herbivory channels the herbivore community into a more specialistic species assemblage associated with the plant that selects against induced plant responses. Differential feedback of induced defence responses in plants on herbivorous insects may, therefore, impose balancing selection between constitutive and induced defences of plants.

Key words: induced defence, biodiversity, herbivore competition, covariation, early-season herbivory, *Pieris rapae*, *Brassica oleracea*

Introduction

Genetic variation in plant traits is a dominant factor in structuring the composition and diversity of insect communities on individual plants (Dungey et al. 2000; Hochwender and Fritz 2004; Wimp et al. 2005; Poelman et al. 2008a). Because of the close reciprocal interactions with plants, bottom-up effects of plant traits are most profoundly affecting herbivorous insects (Johnson and Agrawal 2005). However, direct effects on herbivorous insects may scale up to community-wide effects including higher trophic levels (Bailey et al. 2006; Gruner and Taylor 2006; Bukovinszky et al. 2008).

Community structure of herbivorous insects may also be determined by horizontal effects. It has been identified that the most important of these effects are mediated through plant responses to herbivory (Kaplan and Denno 2007; Kessler and Halitschke 2007). Herbivorous insects have been found to induce changes in the plant's nutritional, allelochemical or morphological status (Karban and Baldwin 1997). These induced plant responses that follow after damage include, for example, altered leaf toughness, trichome density and concentration of secondary chemicals (Traw and Dawson 2002; Poelman et al. 2008c). Although the altered traits may function as induced resistance when they negatively affect the performance of the attacking organism, they are unavoidably interacting with other subsequently colonizing herbivores (Halitschke et al. 2008). Indeed, herbivore-induced plant responses have been found to affect performance (Agrawal 2000; Traw and Dawson 2002; Poelman et al. 2008b), host plant preference (Shiojiri et al. 2002; Poelman et al. 2008b) and predation risk of subsequently colonizing herbivores (Shiojiri et al. 2001; Kaplan et al. 2007). Furthermore, previous herbivory may modulate plant defence responses to subsequent attackers at the transcriptome level (Voelckel and Baldwin 2004; Viswanathan et al. 2007; Poelman et al. 2008b). Induced plant responses, therefore, are a mechanism through which herbivorous insects may interact, even when they are temporally separated (Denno et al. 1995; Kaplan and Denno 2007). Plant-mediated herbivore interactions in the field have been identified between herbivores of the same feeding guild as well as across feeding guilds (Denno et al. 2000; Kessler and Baldwin 2004; Viswanathan et al. 2005). Often, induced plant responses are reported to negatively affect performance of all subsequent colonizers, although the effects may be asymmetric (Inbar et al. 1999; Agrawal 2000; Denno et al. 2000). More importantly, induced responses of plants may have contrasting effects on host plant acceptance by herbivores (Martinsen et al. 1998; Kaplan and Denno 2007; Poelman et al. 2008b). Generalist herbivores have been found to avoid induced plants and their population densities are reduced, whereas specialists may preferentially colonize induced plants (Martinsen et al. 1998; Long et al. 2007; Kaplan and Denno 2007; Poelman et al. 2008b). Dissimilarity in the preference of specialist and generalist herbivores for induced plants may thereby alter the composition of the local herbivore community, which in turn may translate into plant-associated biodiversity differences (van Zandt and Agrawal 2004). Furthermore, when groups of herbivores within a community react oppositely to induced plant responses, plants achieve resistance against one group but at the same time become susceptible to another group of herbivores. However, there is considerable debate regarding the question whether herbivores with similar degree of host-plant specialization covary in their direction of response (Leimu and Koricheva 2006; Johnson and Agrawal 2007). If we consider induced plant responses from a community perspective it is important to determine whether individual herbivore species or, through covarying reactions, groups of herbivores exert selection on induced plant responses.

Here, we present a field study in which we experimentally tested 1) whether early-season herbivores affect diversity of the subsequently colonizing herbivorous insect community; 2) whether there is covariation in herbivore behavioural responses to induced plant

responses among species belonging to the same category of host plant specialization. We induced *Brassica oleracea* plants early in the season by infesting plants with caterpillars of the Small Cabbage White *Pieris rapae* (Pieridae). The caterpillars were removed after one week and on a weekly basis the development of the herbivorous insect community was monitored on induced versus control plants.

We found that early-season herbivory affected herbivore community diversity and composition over the entire spring and summer growth season. Within both specialist and generalist herbivore groups we found covariation in their response to herbivory-induced plants. On the one hand induced responses resulted in resistance against generalist herbivores, on the other hand these responses resulted in induced susceptibility to specialist herbivores. Induced plant responses consequently drove the herbivore community towards dominance by specialists. The contrasting effects of induced plant responses on herbivores with different degree of host plant specialization and the concomitant trade-off in induced defence responses of plants are discussed.

Material and Methods

Plants and insects

We used two cultivars of white cabbage *Brassica oleracea* var. *alba* L. that differ in resistance against herbivores: the susceptible cultivar Badger Shipper (Centre for Genetic Resources, CGN-Wageningen, The Netherlands), and the resistant cultivar Rivera (Bejo Zaden BV, Warmenhuizen, The Netherlands) (Poelman et al. 2008c). Seeds of the cultivars were sown individually in peat soil cubes (3 x 3 x 3 cm). During the first three weeks of germination and seedling growth, the trays with soil cubes were placed in a greenhouse compartment, providing the plants with a 16/8 (day/night) photoperiod of SON-T light (500 μmol/m^2/sec), 18–26ºC and 40-70% relative humidity. After three weeks the plants were placed outside during the day to adjust plants to field conditions. When five weeks old, plants were transplanted while rooted in their soil cubes into the soil at the experimental site.

For the induction treatment in the field we used *Pieris rapae* L. (Lepidoptera: Pieridae) caterpillars that originated from the stock rearing of the Laboratory of Entomology, Wageningen University. The culture was maintained on Brussels sprouts plants (*Brassica oleracea* var. *gemmifera* L. cultivar Cyrus) in climatized rooms at 20-22ºC, 50-70% relative humidity and a 16/8 hour light/dark photoperiod.

Common garden experiment

We conducted a common garden experiment to test whether early-season herbivory by *P. rapae* affected biodiversity and community composition of phytophagous insects in the field. In week 19 (11 May) in 2006, 40 plots (6 x 6 m), each planted with 49 plants of one of the two cultivars were established in an agricultural field in the vicinity of Wageningen, The Netherlands, using a randomized design. Five-week-old plants were planted in a square of 7 x 7 plants with a spacing of 75 cm between plants. We isolated plots by strips of 6 m that were sown with a grass seed mixture of *Lolium* and *Poa* species. In Week 21 (22 May), we infested all plants in half of the plots per cultivar (n = 10) with two second instar *P. rapae* caterpillars that commonly are the first caterpillars colonizing *Brassica* plants in The Netherlands. The caterpillars fed on the plants for seven days and were removed thereafter. The plants were exposed to naturally occurring herbivore populations. From week 23 (5 June) until week 36 (8 September), the central nine plants of each plot were surveyed weekly by investigating both sides of all their leaves for the presence of herbivorous insects.

Table 1. Herbivore species and their degree of host plant specialization found on the *B. oleracea* plants

Species	Order	Family	Feeding type	Host specificity
Pieris rapae	Lepidoptera	Pieridae	Leaf chewer	Specialist
Pieris brassicae	Lepidoptera	Pieridae	Leaf chewer	Specialist
Plutella xylostella	Lepidoptera	Yponomeutidae	Leaf chewer	Specialist
Evergestis forficalis	Lepidoptera	Crambidae	Leaf chewer	Specialist
Mamestra brassicae	Lepidoptera	Noctuidae	Leaf chewer	Generalist
Autographa gamma	Lepidoptera	Noctuidae	Leaf chewer	Generalist
Brevicoryne brassicae	Hemiptera	Aphididae	Phloem feeder	Specialist
Myzus persicae	Hemiptera	Aphididae	Phloem feeder	Generalist
Other aphids	Hemiptera	Aphididae	Phloem feeder	Generalist
Aleyrodes proletella	Hemiptera	Aleyrodidae	Phloem feeder	Specialist
Phyllotreta undulata	Coleoptera	Chrysomelidae	Leaf chewer	Specialist
Phyllotreta atra	Coleoptera	Chrysomelidae	Leaf chewer	Specialist
Thrips tabaci	Thysanoptera	Thripidae	Cell content feeder	Generalist

Biodiversity calculations

During the season we found eleven species of herbivorous insects (Table 1). Out of these eleven species, we could not accurately count the number of whitefly and thrips without damaging the plants and, therefore, excluded these herbivores from the analysis. For each week, the remaining herbivore data were computed into an average value per herbivore species on plot level by averaging the herbivore count data of the nine plants. These values were used to calculate for each week: (a) the total abundance of herbivores, (b) the herbivore species richness, (c) the Shannon-Wiener Index (H`) and (d) Simpson's diversity Index (1-D). The latter two biodiversity indices describe herbivore diversity by incorporating both the richness of species as well as the evenness of their distribution. Simpsons Index of diversity (D) presents the chance that a random draw of two species from a plot results in two individuals of the same species. The lower the value of D, the higher is the diversity of the sample. For this reason 1-D is often presented so that higher values correspond with higher diversity. The more abundant species contribute more strongly to the Index than rare species. The Shannon-Wiener index also takes into account the species richness and the abundance of each species. Both unique species and higher evenness of their abundance distribution increases the value. These two indices are the most commonly used to describe biodiversity.

Phytochemistry of field plants

To assess whether *P. rapae* feeding at the beginning of the season altered plant defence compound levels, we collected leaf material to measure glucosinolate concentration as a proxy for induced plant responses. In week 24, two weeks after the caterpillars that had been introduced for the induction treatment were removed, we collected two leaf disks of a total of five plants for each plot, using a cork borer (diameter 2.3 cm). The ten leaf disks were pooled per plot and stored on ice. Within two hours from sampling the first plant, the leaf disks were transferred to a -80 ºC freezer. At the end of the season in week 35, we collected leaf material following the same protocol as described above to assess whether the treatment of early season herbivory was still traceable in foliar glucosinolate concentration 13 weeks after induction.

The frozen samples were freeze-dried and ground to a fine powder. Amounts of 100 mg of ground leaf material per sample were dissolved in methanol. The extract was

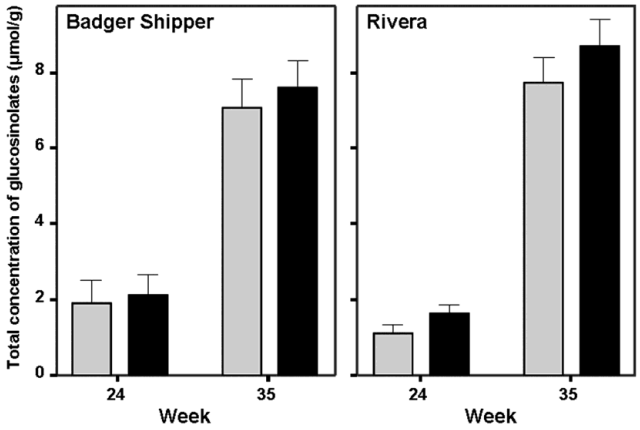

Figure 1. Total foliar glucosinolate concentration (micromoles g^{-1} dry mass + SE) of control plants (grey) and plants induced by early season herbivory by *Pieris rapae* (black) in week 21. Glucosinolate concentration of induced plants were significantly higher than control plants of both cultivars.

desulphatased on a DEAE-Sephadex A25 column and the glucosinolate content was assessed by high performance liquid chromatography (HPLC), using the method described by van Dam *et al.* (2004). Glucosinolate detection was performed with a photodiode array (PDA) detector (200 – 350 nm) with 229 nm as the integration wavelength. A sinigrin (sinigrin monohydrate, ACROS, New Jersey, USA) concentration series was used as an external standard. We used the correction factors at 229 nm from Buchner (1987) and the EC (EC, 1990) to calculate the concentrations of the glucosinolates. Desulfoglucosinolate peaks were identified by comparison of HPLC retention times and UV spectra with standards kindly provided by M. Reichelt, MPI Chemical Ecology, Jena (Germany) and a certified rapeseed standard (Community Bureau of Reference, Brussels, code BCR-367R).

Statistical analysis

Biodiversity: We analysed the development of herbivore abundance, species richness, Shannon-Wiener Index (H`) and the Simpsons Index of diversity (1-D) using structured repeated measurements mixed models. The models were constructed with the PROC MIXED function of SAS 9.1, using a repeated structure type AR(1). We modeled the dependent variable, i.e. biodiversity parameter, including the factors cultivar, week (23-36), treatment (control, induced) and their factorial interactions.

Species abundance: To determine which species in the herbivore communities were affected most by induction treatment or cultivars, we constructed principal response curves (PRC) using the CANOCO software package 4.51 (Ter Braak 1988). The PRC method uses partial redundancy analysis (RDA) and plots the first principal component of the treatment effect against time by contrasting each treatment against a preset control. We used control plants of the cultivar Rivera as the preset control and thus the vertical axis of the PRC diagram shows the contrast of the other treatments, i.e. Badger Shipper control, induced Rivera, induced Badger Shipper, with Rivera control. The PRC method is constrained and extracts information only from the part of the variance that is explained by the environmental factors (cultivar and induction treatment in our case) and implements time (weeks in our case) as a co-variable. We log-transformed the species abundance scores, since high abundance values influence the result of the PRC analysis stronger than low abundance values. Monte Carlo permutation tests with 999 permutations were used to test for significance of the principal component. Associated with the PRC diagram we present the set of species weights on the first principal component. The species weight describes the relative difference of abundance between cultivars and their induction treatment for each herbivore species. A positive weight can be interpreted as a larger abundance of the

particular species on the cultivar that also has a positive PRC score (Lepŝ and Šmilauer 2003).

Response covariance: We addressed whether herbivores with the same host plant range covaried in their response to induced plants and thus whether herbivores with different host plant range (specialists or generalists) had contrary responses. We first performed cluster analysis for the two cultivars separately to visualize how herbivores covaried in their response to induced defenses within cultivars. Cluster analysis creates a dendrogram depicting how herbivores covaried in their abundance over the field plots measured, with all the herbivore species individually depicted on the tips of the dendrogram. The species that link closest with each other in the dendrogram covary strongest in their abundance over field plots. To perform the analyses, we calculated the mean abundance of each herbivore for each of the field plots that were monitored over 14 weeks. We then separated the data obtained for the two cultivars since we were interested in covariation of herbivore responses to induced plants and not in covariation in response to cultivars. The two data sets were each standardized for the abundance of herbivores so that each herbivore species had a mean of zero and a standard deviation of one. The standardized abundance values of each species per field plot were then used in the calculation of the Pearson's correlation matrix (Appendix A). We used the Ward's linkage method to determine the linkage and distance between herbivore species. To test statistically whether herbivores with the same host plant range correlated stronger in their abundance distribution than species with different host plant range, we used an ANOVA test on the Pearson's correlation scores. We tested whether r-values within generalist and specialist herbivores were higher than r-values between these two herbivore groups. In the ANOVA analysis we included r-values between herbivore combinations for both cultivars and included cultivar as a factor in the model to test whether covariation in response of herbivores was different for cultivars.

Results

Plants that had been induced with *P. rapae* caterpillars early in the season had higher foliar concentrations of glucosinolates than control plants (Figure 1) (ANOVA, treatment effect: $F = 8.871$, $P = 0.004$). The total concentration of glucosinolates increased at the end of the season and did not differ between cultivars (Figure 1) (ANOVA, time effect: $F = 1081.233$, $P < 0.001$; cultivar effect: $F = 0.378$, $P = 0.54$; cultivar*treatment $F = 0.939$, $P = 0.34$; cultivar*time interaction effect: $F = 17.844$, $P < 0.001$; treatment*time interaction effect: $F = 1.028$, $P = 0.31$; cultivar*treatment*time interaction effect: $F = 0.019$, $P = 0.89$).

Many of the herbivore species found in the field (Table 1) had two or more generations during the study period. Over time, herbivore abundance, richness and diversity were highest midway the study period and decreased in late summer (Figure 2). Herbivore community development over time was different on the two cultivars and affected by the early season induction treatment (Figure 2, Table 2). In contrast to the expectation that induced plant responses would result in resistance against herbivores, induced plants of both cultivars harboured more herbivores than control plants. Furthermore, the herbivore community on induced plants was characterised by a higher species diversity than on control plants, although the treatment effect on species number was marginally non-significant (Table 2). Badger Shipper plants harboured more herbivores and were richer in species than Rivera plants (Table 2). Thus, early-season herbivory by *P. rapae* resulted in higher herbivore biodiversity than was found on control plants. Badger Shipper plants, which harboured more herbivores, also supported a higher biodiversity than Rivera plants (Table 2).

Not only did plant responses to early-season herbivory by *P. rapae* affect biodi-

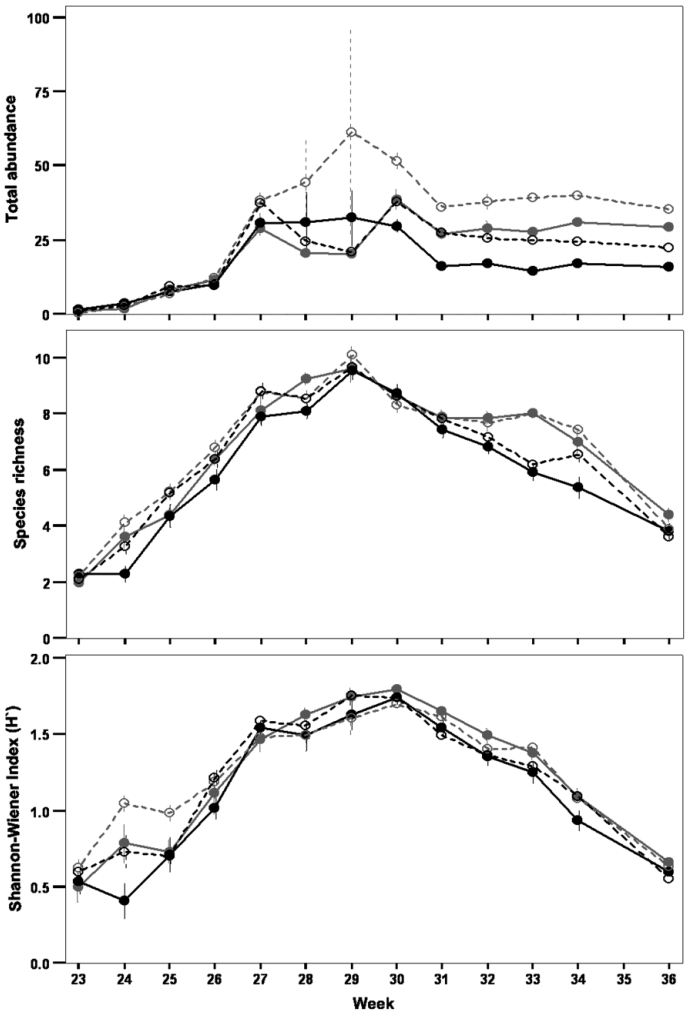

Figure 2. Total herbivore abundance, species richness, and Shannon-Wiener Index (H`) of the herbivore community on two *Brassica oleracea* cultivars over the study period in the field (error bars represent SE); Rivera is a resistant cultivar (black lines) Badger Shipper is a more susceptible cultivar (grey lines). Both cultivars were induced with early-season herbivory by *Pieris rapae*; Induced plants (open circles, dashed line), control plants (closed circles, solid line).

versity on individual plants, the composition of the herbivore community was also strongly affected. The PRC analysis of the contrasts between the cultivars and the induction treatment revealed that herbivore species reacted differentially to early-season herbivory by *P. rapae* (Figure 3) (First RDA axis explained 3.1%, Monte Carlo permutation test F = 84.511, P = 0.002). The species weights on the first PRC axis showed that the specialist herbivores from different feeding guilds, the Cabbage aphid *Brevicoryne brassicae*, the Diamondback moth *Plutella xylostella*, and the two flea beetles *Phyllotreta atra* and *Ph. undulata* were more abundant on plants induced with *P. rapae* herbivory compared to control plants (Figure 3). *Pieris rapae* itself was equally abundant on control and induced plants. Generalist herbivores, such as *Mamestra brassicae* and *Autographa gamma*, were more common on control plants (Figure 3). Cluster analyses for both cultivars supported that generalists and specialists have contrasting reactions to *P. rapae*-induced plants (Figure 4). Within the two herbivore classes, species covaried in their reaction to the induction treatment (ANOVA, host plant range: F = 6.32, P = 0.013) and these reactions were similar in both cultivars (ANOVA, cultivar: F = 1.93, P = 0.168; host plant

Table 2. Cultivar, induction treatment and time effects on herbivore abundance, species richness, Shannon Wiener diversity Index (H`), and Simpson's diversity Index (1-D). For each factor we calculated *F* statistics in Mixed models. Boldface type present significant effects at $P<0.05$.

	Factor					
	Cultivar (1) (d.f. = 1)		Treatment (2) (d.f. = 1)		Time (3) (d.f. = 12)	
	F	*P*	*F*	*P*	*F*	*P*
Abundance	5.19	**0.03**	5.83	**0.02**	149.00	**<0.001**
Species richness	13.29	**<0.001**	2.89	0.09	98.10	**<0.001**
Shannon-Wiener Index (H`)	5.91	**0.02**	2.22	0.15	84.06	**<0.001**
Simpson's diversity Index (1-D)	3.23	0.08	5.02	**0.03**	37.87	**<0.001**

	Interaction							
	1 * 2 (d.f. = 1)		1 * 3 (d.f. = 12)		2 * 3 (d.f. = 12)		1 * 2 * 3 (d.f. = 12)	
	F	*P*	*F*	*P*	*F*	*P*	*F*	*P*
Abundance	0.01	0.93	2.39	**0.005**	1.64	0.08	2.19	**0.01**
Species richness	0.37	0.55	2.31	**0.007**	1.66	0.07	0.74	0.71
Shannon-Wiener Index (H`)	0.83	0.37	2.19	**0.01**	1.41	0.16	0.98	0.47
Simpson's diversity Index (1-D)	0.50	0.48	2.85	**<0.001**	1.89	**0.03**	0.99	0.46

range*cultivar: F = 1.79, P = 0.184) (Appendix A). Thus, early-season herbivory by *P. rapae* drives the herbivore community into a more specialistic species assemblage that is more diverse than the community on control plants. Consequently, induced plant responses resulted in induced resistance to generalists but induced susceptibility to specialist herbivores from different feeding guilds.

Discussion

Early-season herbivory by the specialist *P. rapae* altered glucosinolate concentration in *Brassica oleracea* plants that lasted for at least 13 weeks after the induction treatment. Induced plant responses reduced the number of generalists that were colonizing the plant, but enhanced the abundance of specialist herbivores. The specialists were members of different feeding guilds including the aphid *Brevicoryne brassicae* (phloem sap feeder), the caterpillar *Plutella xylostella* and the two flea beetle species *Phyllotreta atra* and *Ph. undulata* (all three leaf chewers). Similarly, wild radish (*Raphanus raphanistrum*) plants that were damaged by *P. rapae* early in the season attracted *P. rapae* and flea beetles later in the season (Agrawal and Sherriffs 2001), but did not affect generalists such as rabbits. *Pieris rapae*-induced defences are well-known to attract the specialist moth *Pl. xylostella* (Shiojiri et al. 2002; Poelman et al. 2008b). Early-season damage by different herbivore species may have differential effects ranging from no influence at all to strong positive or negative effects on particular herbivore species (Agrawal and Sherrifs 2001; van Zandt and Agrawal 2004; Agrawal 2005; Viswanathan et al. 2005). Evidence is accumulating for a community-wide effect of induced plant responses on both diversity and composition of the plant-associated insect community (van Zandt and Agrawal 2004; Kessler and Halitschke 2007). Induced plant responses after early-season herbivory have been shown to affect herbivore host plant acceptance in a growing number of cases (Kaplan and Denno 2007). In both aquatic and terrestrial systems herbivore interactions are mediated by induced plant responses and early-season herbivory affects the likeliness of future attack for

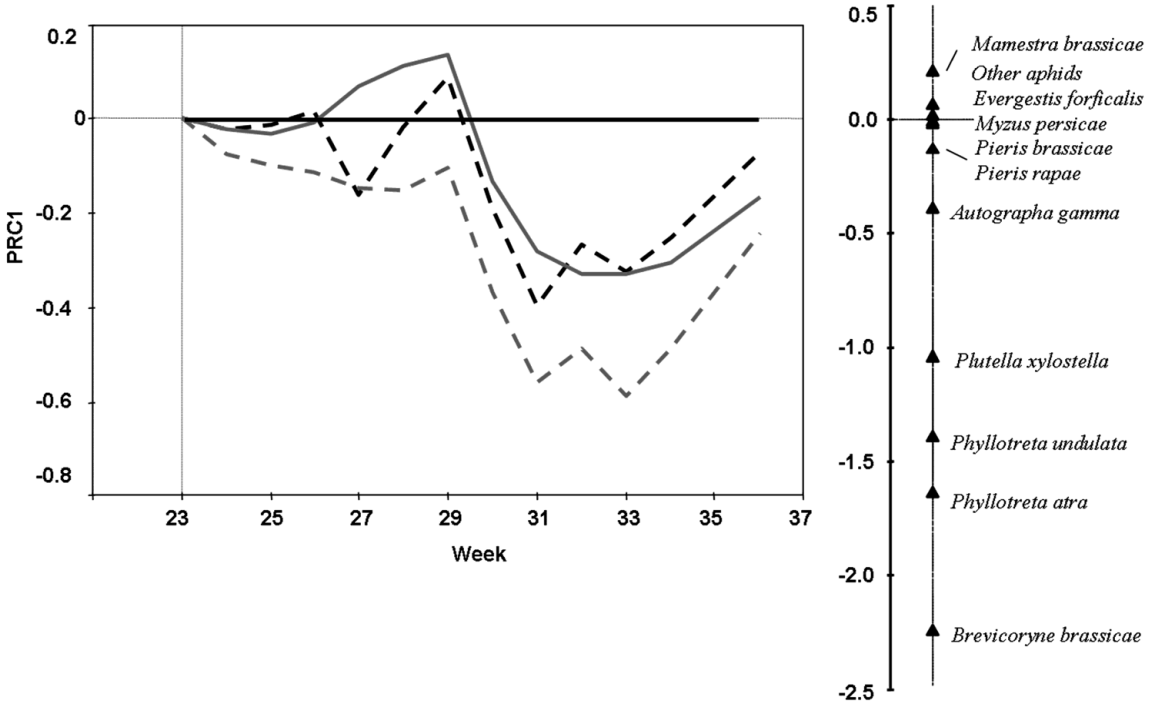

Figure 3. Principal Response Curve (PRC) for herbivore species abundance over time for two cultivars of *B. oleracea* and the early season induction treatment with *Pieris rapae* herbivory. Cultivar differences are related to the control cultivar Rivera (black horizontal reference line). Species weights on the first principal component are depicted in a score plot on the right hand side of the figure. Induction by early season herbi-

a plant by different herbivore species (Denno et al. 2000; van Zandt and Agrawal 2004; Long et al. 2007). Whereas induced responses generally result in induced resistance against generalist herbivores, they may at the same time result in induced susceptibility to specialist herbivores. The outcome of a plant response in terms of community-wide resistance or enhanced susceptibility therefore depends on the community-wide effect of an induced response as well as on the co-variation in reaction by the herbivore community and their natural enemies (Halitschke et al. 2008). Herbivore species clearly influence each other asymmetrically, which raises the question whether groups of herbivores can be identified that are either strong drivers of communities as early-season herbivores or strong responders as late season herbivores.

Strong drivers of herbivore communities alter plant quality in a direction that influences the acceptance of these plants by subsequent species, and therefore can be seen as keystone species in the community (Hunter 1992). Communities develop into a different direction when such species are experimentally removed from the ecosystem or their effect can be measured when applied as early-season inducer of plants. When removing the specialist chrysomelid beetle *Timarcha lugens* from its host plant, Gonzáles-Megías and Gómez (2003) found that the diversity of the herbivore community increased compared to plants that still harboured *T. lugens*. When experimentally inducing plants with either specialist or generalist herbivores it was observed that feeding by specialists strongly affected future community development, whereas effects were less strong or absent for generalist herbivores (van Zandt and Agrawal 2004; Viswanathan et al. 2005). Evidence for covariation in response to induced defences by herbivores with similar host plant range is also

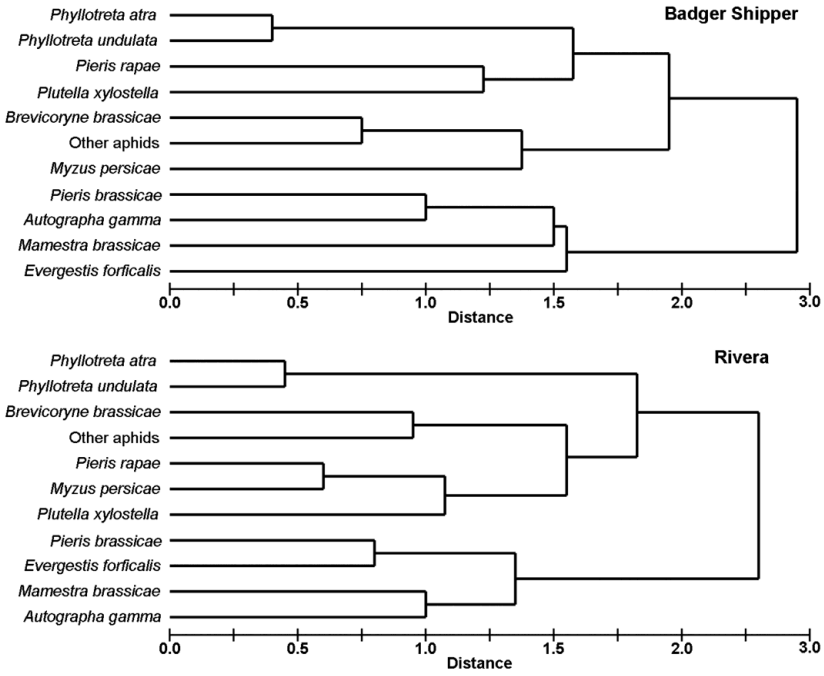

Figure 4. Dendrogram resulting from cluster analysis using Ward's linkage method depicting the covariation of herbivorous insects in abundance among field plots of induced and control plants of two *B. oleracea* cultivars, Badger Shipper and Rivera. Species that link with the lowest distance scores are most similar in their distribution over the field plots.

increasing. Long et al. (2007) found that in the seaweed *Fucus vesiculosus* specialist herbivory suppressed populations of subsequently colonizing generalists, which was caused by a preference of generalists for undamaged seaweed. Other studies supported the avoidance of induced plants by generalists, but more importantly identified a preference of specialists for induced plants (Martinsen et al. 1998; Agrawal and Sherriffs 2001; Shiojiri et al. 2002). Our results show that specialist species exhibited a strong preference for induced plant responses in two cultivars of *B. oleracea*, as indicated by their higher loadings on the first PRC axis (Fig. 3). More importantly, this analysis indicated a contrasting preference of generalists. They were more abundant on control plants. The contrasting preference of generalist and specialist species to induced plant defences was statistically significant and herbivores covaried in response within their category of host plant range. The mechanism underlying the contrasting preference of specialist and generalist herbivores may be found in the exploitation of host plant specific chemical compounds by specialists in host plant acceptance. Generalists typically avoid high concentrations of these compounds (Giamoustaris and Mithen 1995). In brassicaceous plants, glucosinolates are well known to have this contrasting effect on generalist and specialist herbivores (Giamoustaris and Mithen 1995; Gols et al. 2008b). Herbivory elicits a strong, increased production of glucosinolates and may thereby provide specialist herbivores with a stronger oviposition stimulus (van Loon et al. 1992). Induced plant responses to tackle a current herbivore attack may thereby result in future enhanced specialist herbivore attack exerting selection against induced plant responses (Halitschke et al. 2008).

Evolution of induced defence

Constitutive defence, the first line of defence to insect herbivory, can have important implications for the future herbivore community when this defence level differentially attracts generalist or specialist herbivores. Specialists may induce the plant in a way that attracts more specialists; generalists seem not to elicit such responses (van Zandt and Agrawal

2004). Our previous work on constitutive defence of the same cultivars of *B. oleracea* as used here revealed that all herbivores reacted in a similar direction to intraspecific variation in constitutive defence. Both specialist and generalist herbivores colonized plants with low glucoiberin concentration more than plants with high concentrations of this secondary metabolite (Poelman et al. 2008a). However, these results may be caused by relatively low concentrations of glucosinolates in cultivated plants compared to wild congeners (Gols et al. 2008a,b). Constitutive foliar concentrations of glucosinolates have been reported to result in repellence of generalists and attraction of specialists (Giamoustaris and Mithen 1995). High constitutive defence may protect plants from herbivory by generalists, but may increase the probability of specialist attack. Furthermore, maintenance of constitutive defence is costly in the absence of herbivores and may select for induced defences (Karban and Baldwin 1997). However, our present data show that induced defences may have strong contrasting effects on specialists and generalist herbivores. Induced plants suffer a higher attack rate by specialists that may even lead to a higher total abundance and diversity of plant attackers than found on control plants. Furthermore, these specialists such as the aphid *B. brassicae* and the caterpillar *P. xylostella* are members of different feeding guilds (sap suckers versus leaf chewers) and thus demand defence responses of plants on different fronts. On top of that, different feeding guilds induce widely different responses in plants (Heidel and Baldwin 2004; de Vos et al. 2005) that may even work antagonistically on defense responses against attackers from other feeding guilds (Thaler et al. 2002). Plants in a population on which specialist herbivores are dominant may therefore be selected against strong inducible defences, whereas selection pressures may drive towards constitutive defences when generalists dominate the herbivore community. However, induced plant responses may include indirect defence by attraction of predators and parasitoids of the herbivore (Dicke and Sabelis 1988; Turlings et al. 1995; Heil 2008). Indirect defences may thus compensate for the cost of induced susceptibility to specialist herbivores. The entire community including their horizontal species interactions should therefore be considered in estimating the level of selection on the level of constitutive and induced defences (Vrieling et al. 1991).

Induced plant responses structure plant-associated herbivore communities in both terrestrial and aquatic systems (Long et al. 2007). Covariance of herbivore preference for induced plant responses shows that herbivores are not acting as independent sources of selection on plant defences (Dungey et al. 2000; Agrawal 2005; Johnson and Agrawal 2007). Specialist herbivores may drive the herbivore community towards a community dominated by specialist and, thereby, impose selection against induced defences. By contrast, induced defences reduce the attack by generalists that may impose strong selection on the level of constitutive defences. Our results show that generalist and specialist herbivores may thus impose contrasting selection pressures on defence mechanisms and therefore the type of herbivore community may balance plant defence strategies between constitutive or induced mechanisms.

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Appendix A: Pearson's correlation matrix for covariation among the abundance of herbivore species on field plots of two *Brassica oleracea* cultivars Badger Shipper (A), Rivera (B). Abbreviations of species names in the columns correspond with those provided between brackets following the species name in the rows. Bold face type indicates significance $\alpha < 0.05$.

A)		PR	PB	PX	MB	AG	EF	MP	BB	OA	PA	PU
Pieris rapae (PR)	*r*	1	0.11	0.45	**-0.50**	0.08	-0.11	0.01	0.32	-0.01	0.43	0.44
	P		0.68	0.07	**0.04**	0.76	0.69	0.96	0.22	0.96	0.08	0.08
Pieris brassicae (PB)	*r*		1	0.05	0.34	**0.52**	0.70	-0.32	-0.13	-0.27	-0.17	-0.25
	P			0.85	0.19	**0.03**	0.79	0.21	0.62	0.30	0.51	0.33
Plutella xylostella (PX)	*r*			1	**-0.51**	0.27	0.11	0.24	0.27	0.15	**0.50**	**0.48**
	P				**0.04**	0.30	0.69	0.37	0.30	0.57	**0.04**	**0.05**
Mamestra brassicae (MB)	*r*				1	0.33	0.06	-0.28	-0.31	-0.31	**-0.67**	**-0.74**
	P					0.19	0.81	0.28	0.23	0.23	**0.00**	**0.00**
Autographa gamma (AG)	*r*					1	0.25	-0.11	-0.12	0.05	-0.24	-0.28
	P						0.33	0.66	0.65	0.85	0.35	0.27
Evergestis forficalis (EF)	*r*						1	-0.20	-0.10	-0.47	0.19	0.12
	P							0.44	0.71	0.06	0.46	0.64
Myzus persicae (MP)	*r*							1	0.29	0.47	0.07	0.11
	P								0.26	0.06	0.79	0.68
Brevicoryne brassicae (BB)	*r*								1	**0.56**	0.22	0.38
	P									**0.02**	0.40	0.13
Other aphids (OA)	*r*									1	0.07	0.19
	P										0.79	0.46
Phyllotreta atra (PA)	*r*										1	**0.93**
	P											**0.00**
Phyllotreta undulata (PU)	*r*											1
	P											

B)		PR	PB	PX	MB	AG	EF	MP	BB	OA	PA	PU
Pieris rapae (PR)	*r*	1	-0.27	0.25	-0.26	0.29	-0.29	0.35	0.07	0.13	-0.10	0.00
	P		0.22	0.26	0.25	0.20	0.20	0.11	0.74	0.55	0.67	0.99
Pieris brassicae (PB)	*r*		1	-0.28	0.17	0.06	0.30	-0.06	-0.18	-0.23	-0.04	-0.15
	P			0.21	0.46	0.80	0.18	0.80	0.42	0.30	0.87	0.49
Plutella xylostella (PX)	*r*			1	-0.01	0.09	0.10	0.11	-0.05	-0.17	0.19	0.27
	P				0.96	0.70	0.67	0.63	0.81	0.46	0.40	0.23
Mamestra brassicae (MB)	*r*				1	0.25	0.12	-0.08	-0.30	**-0.61**	-0.13	-0.21
	P					0.27	0.59	0.72	0.18	**0.00**	0.56	0.34
Autographa gamma (AG)	*r*					1	-0.22	-0.09	-0.08	-0.34	**-0.62**	**-0.68**
	P						0.32	0.71	0.71	0.12	**0.00**	**0.00**
Evergestis forficalis (EF)	*r*						1	-0.23	-0.06	-0.21	0.32	0.30
	P							0.31	0.78	0.34	0.15	0.18
Myzus persicae (MP)	*r*							1	0.01	0.10	0.14	0.13
	P								0.97	0.66	0.54	0.57
Brevicoryne brassicae (BB)	*r*								1	0.26	-0.08	-0.13
	P									0.24	0.73	0.57
Other aphids (OA)	*r*									1	-0.03	0.07
	P										0.89	0.77
Phyllotreta atra (PA)	*r*										1	**0.93**
	P											**0.00**
Phyllotreta undulata (PU)	*r*											1
	P											

Chapter 8

Picture: Erik Poelman

General discussion

Erik H. Poelman

Introduction

In many ecosystems plants constitute the basal resources sustaining the large diversity of organisms. The fact that plants are consumed by many different types of organisms suggests that there is a strong selection on plants to defend themselves against these attackers. An important premise in Darwinian selection theory is that there is genotypic variation in traits on which natural selection acts and this also counts for plant defensive traits. Plant defensive traits by definition influence in their turn the behaviour, survival and/or reproduction of herbivorous attackers. Not only do plant defensive traits exert selection on higher trophic level organisms, they also contribute to the structure of plant-associated communities (Fritz and Price 1988; Maddox and Root 1990; Dungey et al. 2000; Hochwender and Fritz 2004; Wimp et al. 2005). Genotypic plant variation has been shown to affect the plant-associated insect community more profoundly than environmental factors (Bangert et al. 2006a). However, only few studies identified plant traits that shape insect communities and they predominantly deal with constitutive expression of these traits (Dungey et al. 2000; Johnson and Agrawal 2005; Bailey et al. 2006; Bangert et al. 2006b). Currently debated questions concern whether plant traits affect the composition of insect communities or the abundance of all species equally (Leimu and Koricheva 2006; Johnson and Agrawal 2007) and whether and how different insect species drive a community by affecting each other, as mediated through their effect on the plant's phenotype (Kessler and Halitschke 2007; Bruinsma and Dicke 2008).

The aim of the research programme, in which my thesis project was embedded, was to identify how plant defensive traits structure insect communities. Since plants and their attackers reciprocally affect each other, the programme studied responses of both plants and insects. Studying the role of plant defensive traits in shaping the plant-associated community greatly benefits from a multidisciplinary approach (Kessler et al. 2004; Bruinsma and Dicke 2008; Zheng and Dicke 2008). To measure plant responses to insects, transcriptomics and metabolomics are necessary levels of study. There is ample knowledge on plant responses to single species of herbivores in terms of gene expression or metabolic changes (Heidel and Baldwin 2004; de Vos et al. 2005; Thompson and Goggin 2006). There is, however, limited information on intraspecific variation in plant responses to herbivory on the transcript level (but see Gao et al. 2008). Much less is known about intraspecific variation in plant responses to multiple attackers, which are commonly co-occurring on plants in the field. The solid basis in molecular and chemical plant responses in pair-wise plant-insect interactions provides an opportunity to increase our understanding of the mechanisms of plant responses under the complexity of multiple attackers. At the ecological level there is much more knowledge on the complexity of multi-species interactions (Whitham et al. 2006). It was identified that also plants play an important role in interactions between herbivore species. Species can indirectly influence each other even when they are spatially or temporally separated through mediation by induced changes in plant quality (Faeth 1986; Denno et al. 1995; Kaplan and Denno 2007). To understand the complexity of multi-species associations it is important to understand the effect that each of the species has on a plant and how each of the species responds to herbivore-induced plants.

In this chapter, I discuss the findings of this thesis in comparison with results of the larger research programme it is part of. The programme took a multidisciplinary approach, combining molecular, chemical and ecological aspects of interactions between *Brassica oleracea* and its associated insect community. In this discussion I aim to place the results of my thesis into the wider perspective of the molecular ecology of plant-insect interactions and biodiversity.

Constitutive defences affecting insect communities

Plant genotypes have been documented to widely differ in their constitutively expressed defences that form a first barrier against attackers. Constitutive defences include plant morphological as well as phytochemical characteristics. Genotypic differences in any of these characteristics may affect preference and performance of herbivorous insects and underlie differences in plant-associated insect communities (Johnson and Agrawal 2005; Whitham et al. 2006). Secondary metabolites are well-documented defensive chemicals affecting the abundance and diversity of plant-associated insects (Dungey et al. 2000; Bailey et al. 2006; Bangert et al. 2006b; Berenbaum and Zangerl 2008). In several plant families it has now been identified that plant family-specific chemical compounds negatively affect generalist herbivores, but may positively affect specialist herbivores (Berenbaum and Zangerl 2008). Selection on the concentrations of these compounds may thus be balanced by the type of herbivores that dominate the local herbivore community. Geographic variation in herbivore communities results in different local selection pressures on plant defensive traits. Plant genotypes that have resulted from selection pressures in one locality may spread across other localities and may be differentially accepted by the insect species at this locality than genotypes that have locally evolved. Plant genotypes thereby differ in plant associated insect communities. For example, natural geographic variation as well as variation within populations of brassicaceous plants in secondary chemistry is well known. Along the coastline of England, the naturally occurring *Brassica oleracea* populations widely differ in foliar glucosinolate content (Mithen et al. 1995; Gols et al. 2008a, b). Glucosinolates are the secondary metabolites characteristic for the Brassicaceae and consist of a common chemical structure with a variable side chain, yielding thus far more than 100 different compounds (Fahey et al. 2001). The breakdown of different glucosinolates by myrosinase enzymes results in different products such as isothiocyanates, thiocyanates, nitriles, epithionitriles and oxazolidines (Bones and Rossiter 2006). The intact glucosinolates and their breakdown products have different effectiveness against herbivorous insects (Mewis et al. 2006; Bones and Rossiter 2006). Furthermore, glucosinolates may have opposite effects on generalist and specialist herbivores. Specialist herbivores have a host plant range confined to one plant family or a few phytochemically related families and have adapted to the specific defence chemistry of their host plant (Jaenike 1990; Schoonhoven et al. 2005). Although high concentrations of the chemicals can affect performance of specialist herbivores (Agrawal and Kurashige 2003), the chemicals stimulate host plant acceptance by specialists (van Loon et al. 1992, 2002; Renwick 2002). Generalists are typically deterred by these chemicals and are impeded more in their larval performance than specialists (van der Meijden 1995; Giamoustaris and Mithen 1995; Gols et al. 2008b). Glucosinolates, therefore, seem likely candidates for defensive traits that affect the composition of *Brassica oleracea*-associated insect communities.

A role for constitutively expressed glucosinolate-based defences

Cultivars of *B. oleracea* differ widely in the qualitative composition of foliar glucosinolates, but less in total concentration (Chapter 2; Olsson and Jonasson 1994). The performance of both specialist and generalist herbivores was found to vary among cultivars (Chapter 2; Olsson and Jonasson 1994) but this was not correlated with the total concentration or that of different classes of glucosinolates, i.e. alkenyl-, indolyl- or aromatic glucosinolates (Chapter 2). Neither is the performance of generalist herbivores on the cultivars used here more impeded than the performance of specialists (Chapter 2; Gols et al. 2008b). Plant breeding has selected against the bitter tasting glucosinolates to make brassicaceous plants more acceptable for human consumption, which has resulted in concentrations that

are below an effective threshold against caterpillars (Gols et al. 2008b). Transcriptional responses of cultivars damaged by caterpillars of the Small Cabbage White (*Pieris rapae*) revealed that cultivars differed in transcription of genes encoding glucosinolate biosynthesis but also in genes encoding defensive compounds such as trypsin or protease. Trypsin and protease inhibitors may be more important defensive products against caterpillars in cultivars than glucosinolates (Broekgaarden et al. 2007).

However, even with these low levels of total GS in cultivars, the qualitative differences in foliar glucosinolate composition of the cultivars did correlate with herbivore abundance in the field (Chapter 3). Plants that have glucosinolate profiles in which short side chain compounds were dominant, harboured fewer herbivore numbers and of fewer species than plants with glucosinolate profiles dominated by compounds with longer side chains (Chapter 3). Qualitative differences in glucosinolate profiles of *B. oleracea* cultivars affected herbivore presence, which is supported by oviposition preference experiments of a generalist and specialist herbivore under laboratory conditions (Chapter 3). Thus, the relatively low concentrations of specific glucosinolates in cultivars, while not affecting larval performance, may significantly affect host plant acceptance by adult herbivores. However, the qualitative differences in glucosinolate profiles did not result in contrasting responses of generalist and specialist herbivores. Both groups of herbivores were more abundant on plants that had glucosinolates with long side chains. Particular glucosinolates may, thus, result in resistance to a wide range of herbivores. Although the cultivars of *B. oleracea* also differ in morphological traits such as the number of leaves and biomass, these traits were found not to correlate with herbivore biodiversity (Broekgaarden et al. 2008b). The lack of correlation between plant morphology and herbivore abundance points to phytochemistry as an important determinant of plant-associated herbivore communities. In addition to quantitative differences (Dungey et al. 2000; Johnson and Agrawal 2005; Bailey et al. 2006; Bangert et al. 2006a), qualitative differences in chemical composition of plants play a role in structuring the plant-associated herbivore community (Chapter 3). Although specialists and generalists did not have opposite preferences for cultivars, some herbivores varied more strongly than others in their abundance on the cultivars. The Cabbage aphid *Brevicoryne brassicae* responded most strongly to cultivar variation. Its abundance at the plant level is positively correlated with its performance, which underlies its exponential population growth. The cultivars that harboured a high abundance of Cabbage aphids also sustained high performance in terms of low aphid mortality and highest reproduction rate (Broekgaarden et al. 2008a).

Differences between cultivars in constitutive plant resistance against herbivores thus not only result in differences in herbivore biodiversity, but also in slightly different compositions of the herbivore community. Plant effects on herbivore communities are found to cascade into community-wide effects by affecting predators and parasitoids, as mediated through the herbivore (Bukovinszky et al. 2008). For example, effects on caterpillars may extend to effects on parasitoid performance (Chapter 4). The cultivars that sustained a low performance of *Pieris rapae* caterpillars also resulted in slow development of *Cotesia* parasitoids. However, the ranking of cultivars according to the performance of the herbivorous host did not correspond with their ranking of suitability for wasp development inside these caterpillars. Chemical defences affecting the caterpillar may affect parasitoid larvae and those effects may vary with cultivars (Chapter 4).

Niche availability due to cultivar differences in the number and diversity of herbivore prey affects the abundance and diversity of predators (Bailey et al. 2006). The plant traits affecting herbivores, such as qualitative differences in phytochemistry, therefore are likely to have community-wide effects. The different development of the insect communi-

ties on the cultivars during the season resulted in larger differences between cultivars in induced plant responses later in the growth season than four weeks after planting (Broekgaarden 2008b). Constitutive plant characteristics that affect plant-associated herbivore communities consequently result in differentially induced plant responses through differences in the composition of the herbivore community (Broekgaarden 2008b).

Induced plant responses in a community perspective

A second line of plant defences are those that are expressed in response to attack. These induced defences may benefit the plant by saving costs when attackers are absent and allow plants to accurately direct their defences towards the specific attacker (Karban and Baldwin 1997; Schaller 2008). In the Brassicaceae, plant responses to herbivory include changes in trichome density, thickness of wax layers, concentrations of glucosinolates and production of volatile organic compounds (Geervliet et al. 1997; Traw and Dawson 2002; van Dam 2004). The cultivars used in this thesis differ widely in their response to herbivory as identified by transcriptomic analyses (Broekgaarden et al. 2007, 2008a,b). The response differences include the differential regulation of genes involved in the synthesis of proteinase inhibitors and, glucosinolates as well as volatile terpenoids (Broekgaarden et al. 2007). The cultivars also differed in the degree of increase of indolyl glucosinolate concentration in response to herbivory (Chapter 2). Herbivores themselves have a strong impact on the plant phenotype and thereby they may affect the interaction between plants and other community members. Furthermore, different herbivores have been identified to elicit different transcriptional responses in plants (Heidel and Baldwin 2004; de Vos et al. 2005; Mewis et al. 2006) that result in different concentrations of chemical compounds (Chapter 2) and may thus have differential effects on the development of plant-associated insect communities (van Zandt and Agrawal 2004).

Among herbivore-induced changes the emission of volatile organic compounds functions in the communication with organisms at both the herbivore and higher trophic levels (Halitschke et al. 2008). Herbivore-induced plant volatiles are identified to play an important role in host or prey location by parasitoids and predators (Heil 2008). Volatile cues may be highly specific and provide parasitoids with information on host abundance, species, instar and competitors for their offspring (Blaakmeer et al. 1994; Turlings et al. 1995; Takabayashi et al. 1995; Geervliet et al. 1996; De Moraes et al. 1998; de Boer et al. 2004; Fatouros et al. 2005). When changes in the volatile blend effectively attract parasitoids that reduce the number of plant attackers they serve a role in induced indirect resistance. Plant genotypes differ in their volatile production in response to herbivory and, thereby, affect the abundance and diversity of third trophic level organisms in the field.

The role of volatiles in affecting plant-associated insect communities

The cultivars of *B. oleracea* used here differ in their volatile emission upon herbivory by caterpillars of the Small Cabbage White *P. rapae* (Chapter 4). The differences in herbivore-induced volatile production resulted in in-flight discriminatory behaviour of two congeneric parasitoid wasps in a wind-tunnel assay. The in-flight preference of parasitoids in the laboratory also corresponded with higher fractions of parasitism in the field, indicating that differences in plant volatile production indeed contribute to differences in parasitoid abundance (Chapter 4). By the use of baited traps with volatiles that are known to attract parasitoids and predators, it was established that emission of single compounds could enhance the diversity of predators and parasitoids (James and Price 2004; James and Grasswitz 2005). The released amount of one of these compounds, methyl salicylate, was also found to correspond with the difference in attraction of parasitoids by the cultivars studied

in this thesis (Chapter 4). The differences in parasitoid abundance between cultivars in its turn affect the abundance of species that parasitize cocoons of the primary parasitoids. High primary parasitoid abundance results in high hyperparasitoid abundance and species richness caused by niche availability of primary parasitoid cocoons (Buitenhuis et al. 2005). Furthermore, I demonstrated that the volatile production itself may affect the abundance of hyperparasitoids. Hyperparasitoids were found to discriminate in-flight between caterpillar-infested plants of different cultivars and had the same odour preference for cultivars as the primary parasitoids (Chapter 5). This resulted in higher fractions of hyperparasitism on cultivars that are attractive to primary parasitoids (Chapter 5). Thus, next to cascading effects mediated through herbivore abundance and quality (Bailey et al. 2006; Bukovinszky et al. 2008), herbivory-induced volatiles can directly affect the plant-associated parasitoid community up to the fourth trophic level (Chapter 5).

The two types of plant defence, direct and indirect defence, affect the plant- associated insect community in a relatively independent manner. The cultivars Rivera and Christmas Drumhead that were both more attractive to parasitoids than Badger Shipper (Chapter 4, 5) differed widely in the abundance and diversity of herbivores (Chapter 3). Among the other cultivars, Christmas Drumhead was intermediate in herbivore abundance and diversity, but most attractive to parasitoids. In contrast Rivera sustained relatively few herbivores and was intermediately attractive to parasitoids. Badger Shipper plants were harbouring the highest abundance of herbivores but were least attractive to parasitoids in both the laboratory and in the field. This shows that in these cultivars the defence types are neither mutually exclusive nor directly linked.

The role of induced plant responses in structuring herbivore communities

Herbivore-induced plant responses also result in horizontal effects. Different herbivores feeding on the plant differentially induce plant responses (Agrawal 2000; Traw and Dawson 2002; Chapter 2) and, thereby, differentially affect plant quality for subsequent herbivores. Subsequently feeding herbivores are often negatively affected in their performance by induced plant responses (Agrawal 2000; Kessler and Baldwin 2004; Chapter 6) and herbivores can affect each other asymmetrically (Agrawal 2000). Induced plant responses such as changes in volatile emission or leaf surface compounds may also affect host-plant acceptance by herbivores. In the Brassicaceae, herbivory-induced plant responses consist of an increase in the foliar concentration of indolyl glucosinolates which have been identified to play an important role in herbivore host-plant acceptance (van Loon et al. 1992; Renwick 2002). Glucosinolates stimulate host plant acceptance and oviposition by specialist herbivores, whereas they deter generalist herbivores. Induced increases in glucosinolate concentration thus oppositely affect specialist and generalist herbivores and result in induced resistance to some species and susceptibility to others (van der Meijden 1995; Giamoustaris and Mithen 1995). The specialist herbivore *Plutella xylostella* preferred cultivars that were previously damaged by *P. rapae*, whereas the generalist *Mamestra brassicae* preferred undamaged plants (Chapter 6). Such differences among herbivore species in oviposition preference lead to herbivory-induced differences in community composition of herbivores on plants in the field (van Zandt and Agrawal 2004; Viswanathan et al. 2005; Chapters 6 and 7). Plants that were previously damaged by *P. rapae* in the field harboured more *P. xylostella* but fewer *M. brassicae* caterpillars than control plants (Chapters 6 and 7). Furthermore, across different feeding styles of herbivores, specialist herbivores were more abundant on induced plants than on control plants (Chapter 7). Induced responses to a first colonizing herbivore may thus have community-wide effects for subsequent attackers. The effects of *P. rapae*-induced plant responses on the composition of the herbivore

community were similar for two cultivars. Although these cultivars differed in their full transcriptional responses to *P. rapae* feeding, many of the genes were also induced similarly in both cultivars (Broekgaarden et al. 2007). Plant genotypes may thus differ in their level of constitutive defences and despite differences in their full transcriptional response to herbivory, induced plant responses may converge the communities of different genotypes to become more similar. On two cultivars early season herbivory by *P. rapae* resulted in insect communities that were dominated by specialist herbivores (Chapter 7).

By attracting specialist herbivores from a wide range of feeding styles, plant defensive responses to one herbivore may result in susceptibility to others. Plant responses to each of these subsequent attackers may differ widely because each of the herbivore feeding styles elicits a different type of defence response at the transcriptional level in plants (Voelckel and Baldwin 2004a; Heidel and Baldwin 2004; de Vos et al. 2005). The plant thereby needs to redirect its defences for an accurate response to a subsequent attacker, but this may be constrained by antagonistic effects of signalling pathways (Thaler et al. 2002). On top of that, herbivores themselves may suppress plant responses, thereby further constraining accurate plant defensive responses (Musser et al. 2002; Zarate et al. 2007). Molecular or chemical constraints in defence responses of plants may result in the canalization of defence responses elicited by the first attacker (Voelckel and Baldwin 2004b). The sequence of herbivores inducing the plant affects the plant response (Chapter 6), and thus the induced phenotype. This translates in an even wider array of plant phenotypes that affect subsequent colonization by herbivores (Viswanathan et al. 2007). Differences in transcriptional responses to herbivory among cultivars thus result in differences in community development and those communities in their turn feed back differently on induction of cultivars (Broekgaarden et al. 2008b). Induced plant responses and community reactions thus appear tightly linked through reciprocal effects.

Conclusion

In the past few years the integration between ecology, transcriptomics and metabolomics has resulted in exciting multidisciplinary studies. It is now well established that a multidisciplinary approach addressing different levels of biological organization is needed to understand the interplay between organisms in a dynamic and complex community. Genetic differences in defensive plant traits that can be identified using various molecular tools underlie morphology and metabolic profiles of plants that influence the plant-associated insect community. The wide range of transcriptomic studies in *Arabidopsis thaliana* and its responses to herbivory have identified that plants display different molecular mechanisms to respond to various species of attackers (Heidel and Baldwin 2004; de Vos et al. 2005). In its turn, genetic modification of these traits in *A. thaliana* provided insight into trait-effects on, for example, the performance of herbivores or attraction of predators and parasitoids (Kappers et al. 2005; Schnee et al. 2006). Taking these tools outdoors, genetic modification of specific pathways in plant defence has provided a measure of the impact of single traits on insect communities. *Nicotiana attenuata* plants in which certain genes of the jasmonic acid pathway that is involved in defence response to many herbivores were silenced were commonly attacked by generalist herbivores (Kessler et al. 2004). The silenced plants were even colonized by generalist herbivores that were not known from control *N. attenuata* plants. Furthermore, silenced plants were less capable of attracting predators (Halitschke et al. 2008). A comparison of silenced plants with healthy plants revealed that the volatile alarm call that attracts predators also attracts other herbivores. The studies with plants silenced for a single trait revealed that single plant traits have community-wide effects (Halitschke et al. 2008). Similarly, the use of metabolomic tools resulted in the

identification of specific chemical compounds that affect the abundance of natural enemies. By studying the metabolic profile of different maize cultivars with herbivory by root feeders, Rasmann and collaborators (2005) showed that differences in emission of a specific compound, (*E*)-ß-caryophyllene by the different maize cultivars are responsible for the differential attraction of entomopathogenic nematodes. By the application of this compound to plants in the field the attraction of entomopathogenic nematodes could be enhanced which successfully reduced root herbivory.

In the present research programme, it was demonstrated with the use of molecular tools that herbivore species may elicit different responses in a plant and that plant genotypes also differ in their responses to feeding by the same herbivore (Broekgaarden et al. 2007; Chapter 6). Sequential attack of herbivores even further differentiates plant responses and thereby plant phenotypes (Chapter 6). Responses to early-season herbivory affect the future development of insect communities due to effects on insect host-plant acceptance (Chapters 6 and 7). Induced responses in indirect resistance may affect insect diversity independently of responses in direct resistance. Volatile compounds may promote the abundance of parasitoids and predators without a regulation of their abundance through herbivore community composition (Chapters 4 and 5). In its turn, the community as a whole feeds back to selection on plant defences, meaning that a defence aimed at one species results in altered reactions of other species that together exert the selection pressure on the plant traits. The mechanisms of a particular defence trait, an herbivore-induced plant response or an herbivore's behavioural response have evolved under community constraints and investigating these defences should, therefore, be placed in a community context.

Future directions

Although model systems currently used to study multidisciplinary facets of plant-insect interactions have provided a solid basis for insight into its complexity, there is a need to include natural systems to evaluate the results obtained in model systems. Plant breeding may have deliberately or inadvertently led to altered levels of defence traits that are unnatural. The response of different insect species to variation in cultivars may be different from responses to wild type plants (Gols et al. 2008a,b; Chapter 2). Therefore, in addition to further study plant defence mechanisms using model organisms, the effect of a trait in natural systems should be assessed to quantify its importance under natural conditions. Natural geographic variation in defence traits of *Brassica oleracea* along the coastline of England does provide such a natural system (Mithen et al. 1995; Gols et al. 2008a,b). Furthermore, many of the current field studies on natural systems identify plant traits that correlate with observed effects on insect community composition. However, it remains to be established whether these traits have a causal relationship with the observed effect. Genetic modification of wild type plants will allow assessing the effects of plant defences in a background of naturally selected plant genotypes (Kessler et al. 2004; Wang et al. 2008; Halitschke et al. 2008), although pleiotropic effects of genetic modification of a single trait cannot be ruled out. The targeted modification of specific direct or indirect defence traits allows for a direct assessment of the trait's effect in a community ecological context. Pinpointing the role of specific defence traits or defence pathways will allow addressing central issues in ecology such as whether communities are regulated by top-down or bottom-up effects. The relative role of direct plant defences as a bottom-up factor in structuring communities can be assessed against top-down control that is affected by herbivory-induced volatiles by specifically manipulating traits or pathways involved in these defence types.

Although in Utah, USA, studies on genetically modified wild type plants have provided important insights into the community ecology of plant-insect interactions, legislations restrict the studies to non-flowering plants in the field. Therefore, it is still not possible to assess fitness consequences of defence traits for plants or the interaction of plants with their pollinators under field conditions. Since regulations related to growing of genetically modified organisms in The Netherlands are even more stringent, phenotypic manipulation approaches may provide a sound alternative. The use of exogenously applied elicitors or repressors has proven to provide a useful tool under controlled laboratory conditions (Bruinsma and Dicke 2008) and even in the field (Thaler et al. 2001). However, the use of plant hormones may be less controlled than genetic modification since application of hormones may elicit wider effects in the plant than just the trait of interest.

A second important issue in studies on plant defence is the lack of support for the hypothesis that plant resistance traits also lead to a fitness benefit and can thus be termed defensive traits. Many studies using model systems have been able to identify mechanisms of plant resistance, but did not or could not address whether these mechanisms indeed result in a selective benefit for the plant. It is mandatory to use wild plants in these studies and to measure for example their seed production as a proxy for plant fitness (Smallegange et al. 2008). The progress made in multidisciplinary approaches using model plant systems provides a solid basis for taking the next step to natural ecosystem complexity. The ultimate challenge is to assess the plant fitness benefit of the expression of a particular defence trait when plants are challenged by a diverse community of attackers and synergists that interact amongst each other. Taking these next steps using an integrated approach of molecular, metabolomic and ecological studies will result in a major step forward in our understanding of complex ecological communities.

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A central issue in ecology is to identify the driving forces behind community diversity and the ways in which this biodiversity is maintained. Plant-insect associations form a significant part of the earth's biodiversity and are widely studied for their reciprocal interactions. Plants are under attack by a wide range of organisms that exert selection on plants to defend themselves against these attackers. Genotypes of a plant differ in their defence traits and members of insect communities may respond differently to these genotypes. In chapter 1 of this thesis, studies on plant traits affecting insect communities are reviewed. The review reveals that genotypic variation in plant defence may have a more profound effect on plant-associated insect communities than environmental factors. Genetic variation in plants therefore plays a significant role in structuring and maintaining biodiversity. However, to date only the effect of constitutively expressed direct defence traits has been widely studied with respect to its effect on herbivores and cascading effects on higher trophic levels. Other plant defence traits that interact with higher trophic levels of the insect community as well as inducible defences have not been studied for their effects on plant-associated insect communities. Yet, there is ample knowledge that plants have inducible defences that affect herbivores as well as higher trophic levels in their performance and host plant preference. Induced direct defences include changes in morphology and secondary chemistry that alter plant quality for subsequently colonizing herbivores. Induced indirect defences include emission of volatile organic compounds that attract natural enemies of the herbivores, such as parasitoids and predators.

The aim of this thesis was to identify the effect of intraspecific variation in constitutive direct defence, inducible direct and inducible indirect defences on the diversity and composition of the plant-associated insect community. Cultivars of White Cabbage, *Brassica oleracea*, were used as a model system in conjunction with their insect community. The 85% homology of the genome of *B. oleracea* with the genome of the model plant *Arabidopsis thaliana* allowed a transcriptomic approach by using 70-mer oligonucleotide micro-arrays. Furthermore, as cultivars have been selected for homogeneity, they show little variation within a cultivar, in contrast to the large variation found within wild populations of *B. oleracea*. Cultivars can, therefore, be used as a proxy for genotypic variation in defence traits and can, thus, be studied for differential effects on insect communities. Effects of plant traits on herbivores and parasitoids were studied in the laboratory as well as in the field. I used a multidisciplinary approach that included targeted transcript and metabolic analysis of plant responses to insects as well as an ecological approach to study the effect of plant defensive characteristics on insect communities. In this way the mechanisms underlying the dynamics of ecological communities can be addressed. In Chapter 2, eight White Cabbage cultivars were found to differ widely in their resistance against caterpillars of three different herbivore species, i.e. the specialists *Pieris rapae* and *Plutella xylostella* and the generalist *Mamestra brassicae*. It was hypothesized that the secondary metabolites characteristic for brassicaceous plants, i.e. glucosinolates, were responsible for resistance of plants against the herbivores. In particular, generalist herbivores are known to be affected by these host-plant-family specific defence chemicals. However, the quantitative differences in foliar glucosinolate concentrations of the cultivars did not correlate with the performance of any of the herbivores. The performance of the generalist herbivore *M. brassicae* on cultivars with high glucosinolate concentrations was affected to a similar degree as that of the specialist herbivore *P. xylostella*. Thus, other differences than foliar glucosinolate content caused the resistance differences of the cultivars against these herbivores. Based on the observed differences in herbivore performance, two herbivore-resistant and two herbivore-susceptible cultivars were selected to further study the impact of variation in plant resistance on insect communities.

Effect of constitutive defence on herbivore communities
In Chapter 3 a field experiment is presented in which the two *B. oleracea* cultivars with high herbivore resistance and two with low herbivore resistance were exposed to natural colonization by insects in the vicinity of Wageningen, The Netherlands. Herbivore abundance and diversity were low on herbivore-resistant cultivars. Higher biodiversity was found on cultivars that were more susceptible to herbivory. The qualitative differences and not quantitative differences in foliar glucosinolate profiles between cultivars correlated with the abundance and species richness of the herbivore community. Plants that had high concentrations of the short-side-chain compound glucoiberin harboured low herbivore abundance. When glucosinolate profiles were dominated by compounds with elongated side chains, which are biosynthetically linked to glucoiberin, the plants harboured more herbivore species. Although glucosinolates are known to have differential effects on generalist and specialist herbivores, all herbivores covaried in the direction of response to the intraspecific variation in foliar glucosinolate profiles of the *B. oleracea* cultivars. Furthermore, in laboratory studies both the specialist herbivore *P. rapae* and the generalist herbivore *M. brassicae* preferred to oviposit on cultivars with low glucoiberin concentrations. These laboratory studies, therefore, support the observations on relative herbivore abundance in the field. The results show that qualitative differences in glucosinolate profiles affect the herbivore community associated with brassicaceous plants.

Effect of induced indirect defence on parasitoid communities
The data presented in Chapters 4 and 5 show that plant traits involved in indirect defence affect the community composition of parasitoids associated with the plant. The four cultivars investigated differ in the composition of the volatile blend induced by *P. rapae* herbivory. In wind tunnel tests performed in the laboratory, the two congeneric primary parasitoid wasps *Cotesia glomerata* and *C. rubecula* preferred the volatile blends from the cultivars that were emitting higher concentrations of methyl salicylate and methyl thiocyanate. In the field, caterpillars on the more attractive cultivars were parasitized more frequently than caterpillars on plants that were less attractive to parasitoids. Furthermore, in the field the cocoons of the two *Cotesia* species were more commonly parasitized by hyperparasitoids on cultivars that were more attractive to *Cotesia* wasps. The higher abundance of primary parasitoids only partially explained the higher abundance of hyperparasitoids. Also the behavioural responses of hyperparasitoids to herbivore-induced plant volatiles affect their abundance on the different cultivars. The hyperparasitoid species *Lysibia nana* and *Pteromalus semotus* show the same in-flight preference for herbivore-induced volatiles from the cultivars as primary parasitoids. This demonstrates that herbivore-induced volatiles also directly affect the abundance of hyperparasitoids. Traits involved in indirect plant defences thus directly affect the composition of the parasitoid community in a way that is relatively independent of effects mediated through herbivore communities.

Effect of early-season herbivory on the herbivore community
In Chapters 6 and 7 I show that primary feeding by *P. rapae* not only induced volatile production in *B. oleracea* cultivars that attracted parasitoid wasps, but also affected subsequent colonization by herbivores in multiple ways. *Pieris rapae*-induced responses strongly interacted with plant responses to secondary herbivory. Primary herbivory by *P. rapae* induced the expression of a gene encoding a trypsin inhibitor and a gene that encodes an enzyme involved in glucosinolate biosynthesis. When *P. xylostella* or *P. rapae* were feeding as secondary herbivore, plants responded in a more pronounced way with the expression of defence-related genes than when these herbivores were damaging the plant

as primary herbivore. However, when the generalist *M. brassicae* was feeding on the plant as secondary herbivore the expression levels of defence-related genes were lower compared to expression levels in response to primary herbivory by this species. Initial herbivore-induced responses strongly interacted with plant responses to secondary herbivory and these responses were different for different secondary herbivore species.
The plant responses to *P. rapae* feeding reduced the performance of all three caterpillar species (*P. rapae, P. xylostella, M. brassicae*) compared to their performance when feeding on undamaged plants. However, adult females of the three species had contrasting oviposition preferences for induced plants. *Pieris rapae* itself did not discriminate between plants that were damaged by their larvae and undamaged plants. However, the specialist *P. xylostella* preferred to oviposit on plants that were damaged by *P. rapae*, whereas the generalist *M. brassicae* preferred to oviposit on undamaged plants. Contrasting responses of generalist and specialist herbivores to *P. rapae*-induced plants were also recorded for naturally occurring insects in field studies. Specialist herbivores of different feeding guilds, such as the two leaf-chewing flea beetles *Phyllotreta undulata* and *P. atra* and the phloem feeding Cabbage Aphid, *Brevicoryne brassicae*, were more abundant on *P. rapae*-induced plants than on non-induced plants. The plant responses to a first colonizer resulted in induced resistance to generalist herbivores, but induced susceptibility to specialist herbivores. Consequently, insect communities on induced plants were dominated by specialist herbivores. Moreover, the community on induced plants consisted of more species than found on control plants.

The data presented in this thesis show that intraspecific variation in direct and indirect defensive traits of plants affects the plant-associated insect community. Furthermore, interactions between herbivores mediated through induced plant defences also affect the composition of an insect community. Therefore, the insect community as a whole should be considered in studies of reciprocal selection between insects and defensive traits of plants. A multidisciplinary approach in such studies is of great benefit to gain novel insights into the complexity of plant-insect interactions and the underlying mechanisms.

Binnen ecologisch onderzoek staat de studie van het ontstaan en dynamiek van biodiversiteit in een levensgemeenschap centraal. Planten en de daarvan afhankelijke insecten vormen een belangrijk deel van de biodiversiteit op aarde. De interacties tussen planten en insecten zijn dan ook veelvuldig bestudeerd. Planten worden aangevallen door een grote diversiteit aan organismen die een selectiedruk uitoefenen op de plant om zich tegen deze belagers te verdedigen. Binnen een plantensoort is er genetische variatie in eigenschappen die bijdragen aan de verdediging tegen insecten. Bovendien reageren de verschillende soorten insecten binnen een levensgemeenschap verschillend op de deze variabele eigenschappen van planten. Dit betekent dat genetische variatie in planten leidt tot variatie in de samenstelling van de leefgemeenschap van organismen die aan planten gerelateerd zijn. Hoofdstuk 1 van dit proefschrift geeft een overzicht van de literatuur met betrekking tot de invloed van plantkenmerken op de levensgemeenschap van insecten die op planten leven. Het overzicht laat zien dat genetische variatie binnen een plantensoort een groter effect kan hebben op de soortensamenstelling van insecten dan omgevingsfactoren en dus speelt genetische variatie van planten een belangrijke rol in het behoud van insectenbiodiversiteit. Verreweg de meeste studies hebben gekeken naar verdedigingskenmerken van planten die onafhankelijk van insectenvraat aanwezig zijn. Hieruit blijkt dat er effecten zijn op de levensgemeenschap van plantenetende insecten en ook dat er via de planteneters effecten zijn op de aanwezigheid van predatoren. Veel studies hebben laten zien dat de expressie van verdedigingskenmerken van planten door insectenvraat gestimuleerd kan worden en dat dit andere insecten kan beïnvloeden in hun larvale groei of acceptatie van de plant als voedsel of eileg-substraat. Toch zijn de effecten van deze vraatgestimuleerde verdedigingskenmerken op de diversiteit van insecten niet of nauwelijks bestudeerd. De door insecten geïnduceerde verdediging van planten omvat veranderingen in morfologische kenmerken en de productie van chemische stoffen die resulteren in een veranderde kwaliteit van de plant voor plantenetende insecten die de plant na inductie koloniseren. Een deel van deze chemische stoffen is vluchtig en wordt door predatoren en sluipwespen gebruikt in het lokaliseren van hun prooi. Dit leidt daardoor tot zogenaamde ‘indirecte’ verdediging van de plant tegen plantenetende insecten.

Het doel van dit onderzoeksproject was om te bepalen wat het effect is van variatie in de directe, geïnduceerd-directe en -indirecte verdediging binnen een plantensoort op de insectengemeenschap. Daarbij werd gebruik gemaakt van gecultiveerde witte kool (*Brassica oleracea*) en de insecten die daarop voorkomen. Het genoom van *B. oleracea* komt voor 85% overeen met het genoom van de zandraket, *Arabidopsis thaliana*, hetgeen het gebruik van de voor *A. thaliana* ontwikkelde 70-mer oligonucleotide micro-arrays mogelijk maakt in onderzoek aan *B. oleracea*. Bovendien heeft de veredeling van *B. oleracea* geresulteerd in een diversiteit aan cultivars die binnen de cultivar geselecteerd zijn op homogeniteit in tegenstelling tot de grote genetische variatie binnen natuurlijke populaties van *B. oleracea*. Daardoor kunnen cultivars gebruikt worden als genotypes in studies naar het effect van planteigenschappen op de diversiteit van insecten. Effecten van planteneigenschappen op herbivoren en sluipwespen werden in het veld en in het laboratorium bestudeerd. Daarbij werd een multidisciplinaire benadering gevolgd door gebruik te maken van (1) moleculair-genetische en chemische analyses van de effecten die insecten uitoefenen op plantenverdediging en (2) een ecologische analyse van de effecten van planteigenschappen op de insectendiversiteit. Hierdoor kon worden bestudeerd hoe mechanismen van plantenverdediging de dynamiek van een ecologische levensgemeenschap beïnvloeden. In hoofdstuk 2 werd aangetoond dat acht witte kool cultivars verschillen in resistentie tegen rupsen van drie herbivore insecten, te weten de specialisten *Pieris rapae* (kleine koolwitje) en *Plutella xylostella* (koolmotje) en de generalist *Mamestra brassicae* (kooluil). De hypo-

these dat glucosinolaten, de secundaire plantenstoffen kenmerkend voor Brassicaceae, bepalend zijn voor de resistentie van de planten werd getoetst. De generalistische rupsen werden negatief beïnvloed door hoge concentraties van deze waardplant-specifieke verdedigingsstoffen. Er werd echter geen correlatie gevonden tussen de concentratie van glucosinolaten in de bladeren en de groeisnelheid van de rupsen. Ook reageerden rupsen van de generalist *M. brassicae* en van de specialist *P. xylostella* op vergelijkbare manier op de variatie in plantkwaliteit. Hieruit werd geconcludeerd dat niet de concentraties van glucosinolaten in de bladeren maar andere verschillen tussen cultivars de verschillen in resistentie van cultivars tegen planteneters veroorzaken. De groeisnelheid van de rupsen werd als criterium gebruikt om twee resistente cultivars en twee vatbare cultivars te selecteren voor verdere studie naar het effect van resistentie op de levensgemeenschap van insecten.

Effecten van directe plantenverdediging op herbivoren-diversiteit
In hoofdstuk 3 werden de twee resistente en twee vatbare *B. oleracea* cultivars blootgesteld aan natuurlijke kolonisatie door insecten in een proefveld nabij Wageningen. Op de resistente cultivars werden gedurende het seizoen minder herbivoren gevonden, die behoorden tot een kleiner aantal soorten, dan op de twee vatbare cultivars. De kwalitatieve samenstelling van glucosinolaten en niet de totale concentratie van glucosinolaten in de cultivars correleerde met parameters die insecten-diversiteit kwantificeren. Op planten met een hoge concentratie van glucosinolaten met een korte alifatische keten, zoals glucoiberine, kwamen lage aantallen herbivoren voor. Wanneer in de bladeren juist glucosinolaten met langere alifatische ketens domineerden, die biosynthetisch voortkomen uit stoffen met een korte keten, werden op de planten hogere aantallen insecten waargenomen. Hoewel bekend is dat glucosinolaten een verschillend effect hebben op specialistische en generalistische herbivoren, gedroegen alle herbivoren zich vergelijkbaar in reactie op de variatie in kwalitatieve samenstelling van glucosinolaten in de cultivars. Bovendien legden zowel de specialist *P. rapae* als de generalist *M. brassicae* hun eieren bij voorkeur op planten met lage concentraties glucoiberine in laboratorium-experimenten. De resultaten van de laboratorium-experimenten stemmen overeen met de waarnemingen aan de populatiegrootte van plantenetende insecten op de cultivars in het veld. Dit toont aan dat kwalitatieve verschillen in glucosinolaten de samenstelling van de insectengemeenschap op koolplanten beïnvloedt.

Effecten van indirecte verdediging op de levensgemeenschap van sluipwespen
De resultaten gepresenteerd in hoofdstukken 4 en 5 laten zien dat ook variatie in geïnduceerde indirecte verdediging van planten de soortensamenstelling van sluipwespen op een plant kan beïnvloeden. De vier cultivars die verschillen in resistentie tegen herbivoren bleken ook te verschillen in de vluchtige stoffen die de planten produceerden na vraatschade van *P. rapae*. In windtunnel-toetsen in het laboratorium werd gevonden dat de twee sluipwespen van *P. rapae, Cotesia glomerata* en *C. rubecula*, een voorkeur hadden voor de geuren van cultivars die hogere concentraties methyl salicylaat en methyl thiocyanaat produceerden. In het veld werden rupsen op deze aantrekkelijke cultivars vaker geparasiteerd door de sluipwespen dan rupsen op cultivars die minder aantrekkelijk waren voor sluipwespen. Bovendien werden de cocons van de twee sluipwespen op aantrekkelijke cultivars vaker geparasiteerd door hyperparasitoïden dan cocons op minder aantrekkelijke cultivars. Dit effect zou deels verklaard kunnen worden door de hogere aanwezigheid van cocons op aantrekkelijke cultivars, maar ook het gebruik van dezelfde vluchtige stoffen door hyperparasitoïden zou een verklarende factor kunnen zijn. De voorkeur in windtunnel-toetsen van twee hyperparasitoïden, *Lysibia nana* en *Pteromalus semotus*, voor dezelfde cultivars

als de primaire *Cotesia* sluipwespen bevestigde dit. Dit experiment laat zien dat vluchtige stoffen die vrijkomen na vraatschade van plantetende insecten een direct effect hebben op de aanwezigheid van hyperparasitoïden. Planteigenschappen betrokken bij indirecte verdediging hebben dus een direct effect op de samenstelling van de levensgemeenschap van sluipwespen, deels onafhankelijk van effecten van planteigenschappen op de levensgemeenschap van plantetende insecten.

Effecten van herbivorie op de diversiteit aan herbivoren

In hoofdstukken 6 en 7 laat ik zien dat vraatschade van *P. rapae* niet alleen leidt tot de inductie van vluchtige stoffen die sluipwespen aantrekt, maar ook op verschillende manieren de plantenetende insectengemeenschap beïnvloedt. De inductie van plantenverdediging door *P. rapae* rupsen beïnvloedde de reactie van planten op vraatschade van een tweede herbivoor. *Pieris rapae*-vraat induceerde de expressie van genen die coderen voor de aanmaak van trypsine-remmers en van een enzym dat betrokken is bij de synthese van glucosinolaten. Wanneer de specialisten *P. xylostella* of *P. rapae* daarna op de geïnduceerde planten werden geïntroduceerd, reageerde de plant met een sterkere expressie van deze genen dan wanneer de twee rupsen als eerste vraatschade aan de plant toebrachten. Echter, wanneer de generalist *M. brassicae* als tweede herbivoor vraatschade veroorzaakte, dan was de expressie van deze genen minder sterk dan wanneer *M. brassicae* een onbeschadigde plant aanvrat. De respons van een plant op een eerste belager beïnvloedde de respons van de plant op een tweede belager en die respons was afhankelijk van de identiteit van de tweede belager. Rupsen van de drie soorten (*P. rapae, P. xylostella* en *M. brassicae*) groeiden langzamer op planten die door *P. rapae* waren beschadigd in vergelijking met onbeschadigde planten. Echter, de drie herbivoren verschilden in hun eileg-voorkeur voor beschadigde en onbeschadigde planten. *Pieris rapae* vlinders maakten geen onderscheid tussen onbeschadigde planten en planten beschadigd door rupsen van dezelfde soort. De specialist *P. xylostella* legde meer eieren op planten die geïnduceerd waren door *P. rapae*-vraatschade dan op onbeschadigde planten, maar de generalist *M. brassicae* legde juist meer eieren op onbeschadigde planten. De tegengestelde voorkeur van specialistische en generalistische plantenetende insecten voor geïnduceerde planten werd ook in het veld gevonden. Bovendien bleek dat veel van de specialisten, die sterk verschillen in de manier waarop ze van een plant eten, een voorkeur hadden voor geïnduceerde planten. Zowel de bladvretende rupsen van *P. xylostella*, de aardvlooien *Phyllotreta undulata* en *P. atra*, als de floeemsap-zuigende bladluizen van *Brevicoryne brassicae* waren algemener op geïnduceerde planten dan op controle planten. De geïnduceerde respons van planten resulteerde in sterkere resistentie tegen generalisten, maar tegelijkertijd in een verminderde resistentie tegen specialisten. Dit had tot gevolg dat de levensgemeenschap van insecten op geïnduceerde planten gedomineerd werd door specialistische plantenetende insecten. Bovendien bestond de levensgemeenschap van planteneters op de geïnduceerde plant uit meer individuen van meer verschillende soorten dan op controle planten.

De bevindingen gepresenteerd in dit proefschrift laten zien dat variatie in directe en indirecte verdediging binnen een plantensoort effect heeft op de samenstelling van de levensgemeenschap van de met de plant geassocieerde insecten. Bovendien hebben herbivoren daarbij door hun inductie van plantenverdediging zelf ook een effect op de diversiteit en typen insecten die op een plant worden gevonden. Uit deze resultaten moet worden opgemaakt dat de insectengemeenschap als geheel moet worden meegenomen in studies van plantenverdediging en gedrag van insecten. Een multidisciplinaire benadering in studies van plant-insect interacties geeft daarbij een nieuw inzicht in de onderliggende mechanismen van plantverdediging in de context van de complexiteit van een levensgemeenschap.

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De reizen naar Zuid-Amerika hebben me de nodige ontspanning gebracht om weer de schouders te kunnen zetten onder een veldseizoen of het schrijven van een hoofdstuk. Jan en José Verkade, ik heb ontzettend genoten van de reizen en de gezelligheid in jullie huis in Ecuador. Jan, jouw kennis van de Ecuadoriaanse kikkertjes maakt onderzoek in Ecuador bijzonder efficiënt. Renée van Wijngaarden en Ciska, onze laatste reis naar Nouragues Frans Guyana, is onvergetelijk. Het was fijn om terug te zijn op voor mij de mooiste plek op aarde en dat met jullie te kunnen delen. Marcel, ook bij mijn werk aan gifkikkertjes ben je ontzettend belangrijk geweest. Allereerst door het bieden van de mogelijkheid om bij Entomologie een afstudeervak aan gifkikkers uit te voeren. Dit is het begin geweest van het kikkeronderzoek waar ik met ontzettend veel plezier nog steeds in mijn vrije tijd aan werk. Bedankt voor al je energie die je gestoken hebt in de beursaanvragen en manuscripten. Here I also would like to thank all my colleagues in projects on poison frogs: Philippe Gaucher, Philippe Kok, Luis Coloma, Jason Brown, Kyle Summers, Stefan Lötters, Juan Carlos Santos, Max Ringler, Walter Hödl and Miguel Vences; thanks for sharing ideas and stimulating me to continue my work on frogs.

En mijn familie en vrienden wil ik graag bedanken voor hun steun. Pa en Ma, jullie aandeel is al achtentwintig jaar groots. Ik bof maar met jullie!

Erik

Erik H. Poelman was born on April 21, 1980 in Arnhem, The Netherlands. After finishing secondary school, he started his study biology at Wageningen University in 1998 and obtained his MSc degree in 2003. The subject of his MSc-thesis was space use and social structure of Roe deer (*Capreolus capreolus*), under supervision of Dr. Sip van Wieren (Resource Ecology, Wageningen University). Meanwhile he wrote a research proposal for a study on reproductive strategies of Amazonian poison frogs (*Dendrobates ventrimaculatus*) in collaboration with Prof. Marcel Dicke. In 2002 the proposed study was carried out during a stay of five months at the campsite "Nouragues" in the tropical rainforest of French Guiana, South America. As a final internship in the group of Dr. Kate Lessells at the Netherlands Institute for Ecology (NIOO) in Heteren, Erik joined ongoing research investigating food provisioning behaviour of Great tit (*Parus major*) parents to their offspring. In 2003 he continued working at NIOO as an assistant of Dr. Kate Lessells, studying parent and offspring behaviour in nests of Great tits to elucidate parent-offspring- and sexual-conflict paradigms. In the first half of 2004 Erik worked as a teaching-assistant of evolutionary ecology at the University of Utrecht. In June of 2004, he started his PhD in the group of Prof. Marcel Dicke and Dr. Joop van Loon at the Laboratory of Entomology, Wageningen University, in collaboration with Prof. Louise Vet and Dr. Nicole van Dam at NIOO. His study addressed whether and how intraspecific variation in defence mechanisms of plants affects biodiversity of insects. The results of this research are described in this thesis. Besides his research on plant-insect interactions, Erik continued studying poison frog ecology, in particular their diversification of reproductive strategies and mimetic radiation. These projects were conducted in French Guiana and Ecuador. After defending his PhD dissertation, Erik will work on plant-mediated competition between parasitoid wasps as a post-doc at the Laboratory of Entomology, Wageningen University.

Publications

Poelman, E.H., van Loon, J.J.A., Dicke, M. (2008) Consequences of variation in plant defence for biodiversity at higher trophic levels. *Trends in Plant Science* (in press)

Broekgaarden, C., **Poelman, E.H.,** Steenhuis-Broers, M.M., Voorrips, R.E., Dicke, M., Vosman, B. (2008) Responses of *Brassica oleracea* cultivars to infestations by the aphid *Brevicoryne brassicae*: an ecological and molecular approach. *Plant, Cell and Environment* (in press)

Poelman, E.H., Broekgaarden, C., van Loon, J.J.A., Dicke, M. (2008) Early season herbivore differentially affects plant defence responses to subsequently colonizing herbivores and their abundance in the field. *Molecular Ecology* 17:3352-3365

Poelman, E.H., Galiart, R.J.F.H., Raaijmakers, C.E., van Loon, J.J.A., van Dam, N.M. (2008) Performance of specialist and generalist herbivores feeding on cabbage cultivars is not explained by glucosinolate profiles. *Entomologia Experimentalis et Applicata* 127:218-228

Poelman, E.H., Dicke, M (2008) Space use of Amazonian poison frogs: testing the reproductive resource defense hypothesis. *Journal of Herpetology* 42:270-278

Broekgaarden, C., **Poelman, E.H.,** Steenhuis-Broers, M.M., Voorrips, R.E., Dicke, M., Vosman, B. (2007) Genotypic variation in genome-wide transcription profiles induced by insect feeding: *Brassica oleracea – Pieris rapae* interactions. *BMC Genomics* 8:239

Poelman, E.H., Dicke, M. (2007) Offering offspring as food to cannibals: oviposition strategies of Amazonian poison frogs (*Dendrobates ventrimaculatus*). *Evolutionary Ecology* 21:215-227

Lessells, C.M., **Poelman, E.H.,** Mateman, A.C., Cassey, P. (2006) Consistent feeding positions of Great tit parents. *Animal Behaviour* 72:1249-1257

Kok, P.J.R., MacCulloch, R.D., Gaucher, P., **Poelman, E.H.**, Bourne, G.R., Lathrop, A., Lenglet, G.L. (2006) A new species of *Colostethus* (Anura: Dendrobatidae) from French Guiana with a redescription of *Colostethus beebei* (Noble, 1923) from its type locality. *Phyllomedusa* 5(1):43-66

Submitted:

Poelman, E.H., van Dam, N.M., van Loon, J.J.A., Vet, L.E.M., Dicke, M. Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores.

Poelman, E.H., van Loon, J.J.A., van Dam, N.M., Vet, L.E.M., Dicke, M. Community-wide effects of induced plant responses: a trade-off between induced resistance to generalist and susceptibility to specialist herbivores.

Broekgaarden, C., **Poelman, E.H.,** Voorrips, R.E., Dicke, M., Vosman, B. Intraspecific variation in herbivore community composition and transcriptional profiles in field-grown *Brassica oleracea* cultivars.

Poelman, E.H., Oduor, A.M.O., Broekgaarden, C., Hordijk, C.A., Jansen, J.J., van Loon, J.J.A., van Dam, N.M., Vet, L.E.M., Dicke, M. Field parasitism rates of caterpillars on *Brassica oleracea* plants are reliably predicted by differential attraction of *Cotesia* parasitoids.

In preparation:

Poelman, E.H., Boursault, A.E., Jongema, Y., van Loon, J.J.A., Harvey, J.A., Vet, L.E.M., Dicke, M. Plant variation in herbivore-induced volatiles affects hyperparasitoid attraction and hyperparasitoid community composition.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Erik H. Poelman
Date: 1 October 2008
Group: Laboratory of Entomology, Wageningen University

1) Start-up phase	*date*
► **First presentation of your project**	
Linking variation in plant defense to higher trophic level biodiversity	Dec 01, 2004
► **Writing or rewriting a project proposal**	
► **Writing a review or book chapter**	
Consequences of variation in plant defense for biodiversity at higher trophic levels	Mar 2008
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
Radboud University Nijmegen	Jun 02, 2005
Wageningen University	Sep 19, 2006
Wageningen University	Sep 13, 2007
► **EPS theme symposia**	
Theme 2 'Interactions between Plant and Biotic agents', Utrecht University	Sep 17, 2004
Theme 2 'Interactions between Plant and Biotic agents', University of Amsterdam	Feb 02, 2007
► **NWO Lunteren days and other National Platforms**	
Signalling in Biology, Leiden University	Sep 01, 2004
Netherlands Ecological Research Network 1st Annual Meeting, Lunteren	Feb 12-13, 2008
► **Seminars (series), workshops and symposia**	
TOE symposium 'Investigating the genetics of natural variation', Wageningen, NL	Nov 01, 2004
Workshop 'Plant-Insect interactions: from mol. Biology to ecology', Wageningen, NL	Apr 25, 2006
Workshop 'Plant-Insect interactions: from mol. Biology to ecology', Amsterdam, NL	Oct 26, 2007
► **Seminar plus**	
► **International symposia and congresses**	
VOCBAS Workshop: Improving the interpretation and application of plant-volatile analysis, Wageningen, NL	Mar 15-18, 2006
IOBC-Doorn, The Netherlands	May 11-14, 2007
SIP-13, Uppsala, Sweden	Jul 29-Aug 02, 2007
► **Presentations**	
Multitrophic Interactions, KNAW-NIOO, Heteren, NL	Dec 01, 2004
IOBC-Doorn	May 11-14, 2007
SIP-13, Uppsala, Sweden	Jul 29-Aug 02, 2007
NERN, Lunteren	Feb 12-13, 2008
► **IAB interview**	Sep 18, 2006
► **Excursions**	
PhD Excursion England	Mar 05-08, 2007
Subtotal Scientific Exposure	*11.6 credits**

3) In-Depth Studies	*date*
► **EPS courses or other PhD courses**	
PhD Course 'Metabolomics'	May 02-04, 2005
Spring School 'Chemical communication: from gene to ecosystem'	Mar 19-23, 2005
Advanced Statistics	Feb 27-Mar 02, 2007
► **Journal club**	
PhD lunch meeting Entomology & MTI, NIOO meeting, Heteren	2004-2008
► **Individual research training**	
Subtotal In-Depth Studies	*6.9 credits**

4) Personal development	*date*
► **Skill training courses**	
English Scientific Writing	Feb 13-Apr 10, 2006
► **Organisation of PhD students day, course or conference**	
Organisation of Workshop 'Plant-Insect interactions from mol. biology to ecology (2006)	Apr 25, 2006
► **Membership of Board, Committee or PhD council**	
member of the PhD student council	2004-2008
Subtotal Personal Development	*4.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**30.7**

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 credits

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

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Cover picture: *Pieris brassicae* by Erik Poelman
The figure on the back of this thesis illustrates an insect community with emphasis on the Lepidoptera that can be found on *B. oleracea*.

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