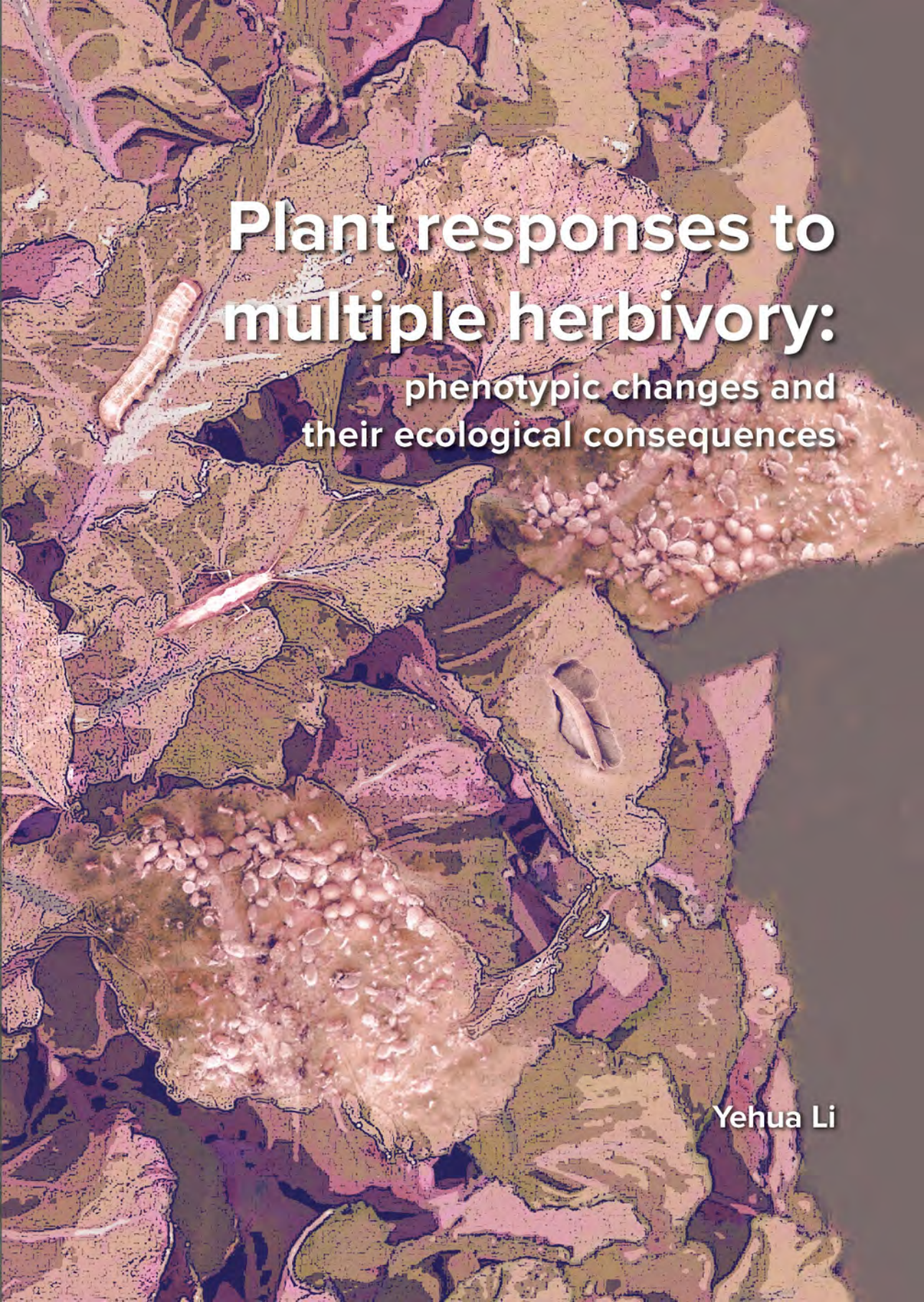

# Plant responses to multiple herbivory:

## phenotypic changes and their ecological consequences

Yehua Li

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# Plant responses to multiple herbivory:
phenotypic changes and their ecological consequences

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# General introduction

Yehua Li

Chapter 1

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Plants live in a hostile environment and are challenged by a diverse range of attackers, including pathogens and insect herbivores that may attack the plant either simultaneously or sequentially. To cope with this diversity of biotic threats that may reduce survival and fitness of plants, they are equipped with traits that prevent or reduce attack by biotic agents. These traits, both physical and chemical, can be constitutively expressed or may be activated or enhanced upon attack (De Vos et al. 2005; Dicke and Baldwin 2010). Constitutively expressed traits may be costly to maintain when herbivores are absent, whereas plant responses induced upon herbivory provide a more efficient strategy for plants to cope with the temporal and spatial variability of herbivory (Karban and Baldwin 1997; Agrawal and Karban 1999). Induced plant responses also represent a type of plant phenotypic plasticity. They can be expressed locally but can also spread systemically to non-infested parts of the plants and can be long-lasting (Agrawal 1999b; Kessler and Baldwin 2002). Herbivore-induced changes in the plant's phenotype may affect other insect herbivore species that attack the plant successively and may also affect higher trophic level organisms that are associated with the host plant, and as a result these changes may have consequences for the insect community in time and space (Agrawal 1999a; Kessler and Halitschke 2007; Stam et al. 2014).

To respond adequately to various biotic threats, plants need to detect and differentiate between different species of attackers (Wu and Baldwin 2009, 2010; Erb et al. 2012). Different herbivore species have been shown to differentially induce phenotypic changes in the plant (Agrawal 2000; Van Zandt and Agrawal 2004b; Viswanathan et al. 2005). Moreover, attack by multiple herbivores, either simultaneous or temporally separated, induces different transcriptomic and metabolic changes in plants compared to single attack (Kessler and Baldwin 2004; Voelckel and Baldwin 2004; Mewis et al. 2005; Mewis et al. 2006; Rodriguez-Saona et al. 2010). Specificity in responses to different attackers allows plants to mount a defence that can more effectively cope with herbivore species with distinct life styles and feeding strategies (Howe and Jander 2008; Pieterse et al. 2009). According to their feeding modes, herbivores can be broadly grouped into leaf chewers and phloem feeders (Schoonhoven et al. 2005). Defences against leaf-chewing and phloem-feeding herbivores are regulated by two major signalling pathways controlled by the phytohormones jasmonic acid (JA) and salicylic acid (SA). Although there are exceptions, leaf-chewing herbivores generally activate defence responses regulated by JA, whereas phloem-sucking herbivores predominantly activate defences regulated by SA (Kessler and Baldwin 2002; Mewis et al. 2005; Howe and Jander 2008; Thaler et al. 2012). When plants are challenged by both phloem-feeding and leaf-chewing herbivores, crosstalk between SA an JA signalling pathways may occur, which may help plants to fine-tune their response to the attackers encountered (Howe and Jander 2008, Pieterse et al. 2009, Stam et al. 2014).

Closely related plant species may vary in their responses to the same type of herbivory (Dungey et al. 2000; Schmidt et al. 2005; Agrawal et al. 2014). Moreover, within one species, heritable variation in resistance traits is an important component in the adaptation of plants to environmental stresses (Wu et al. 2008; Johnson et al. 2009). Intraspecific variation was found for plant secondary metabolites and signal-transduction activation in e.g. brassicaceous plants, tobacco and rice (Kliebenstein et al. 2001; Lou et al. 2006; Gols et al. 2008; Wu et al. 2008; Newton et al. 2009b; Dicke and Baldwin 2010). Genetic diversity in resistance traits among plant individuals and populations can influence the abundance of insect species and the diversity of insect communities (Johnson et al. 2006; Newton et al. 2009a; Agrawal et al. 2012). Phenotypic plasticity in plant responses to herbivory may further shape the composition, diversity and dynamics of the insect community, but previous studies primarily investigated the community-wide effects of induction by leaf-chewing herbivores (Agrawal 1999a; Van Zandt and Agrawal 2004a; Bukovinszky et al. 2010); less is known about community-wide effects of induction by phloem-feeding herbivores.

While trophic interactions involving single species at each trophic level have been intensively studied within the research on plant-insect interactions, the ecological consequences of plant responses to multiple herbivory on subsequent attackers and eventually on the entire insect community are less well explored. Recent studies revealed differential molecular mechanisms underlying plant responses to single herbivory by insects of different feeding guilds, but the elucidation of mechanisms underlying plant responses to multiple herbivory by members of different feeding guilds has only just begun (Voelckel and Baldwin 2004; Zhang et al. 2013). To gain a better understanding of plant interactions with multiple herbivores from molecular mechanism to ecological consequences, it is crucial to use a multidisciplinary approach.

## Main objective and research question

The main objective of my PhD research was to investigate plant interactions with two herbivores belonging to different feeding guilds, i.e. a phloem-sucking and a leaf-chewing herbivore, and how plant intraspecific variation may affect these interactions, with a focus on how aphid infestation may interfere with the plant response to caterpillar attack. I determined the effect of aphid infestation on insect performance and behaviour in the laboratory, and insect community structure and dynamics in the field. At the mechanistic level, I compared plant responses to dual and single herbivore attack by quantifying gene transcript levels of marker genes of the two major defence signalling pathways and by assessing the composition of the volatile blends emitted in response to single and dual herbivore attack. I addressed the following research questions:

1. How does aphid infestation affect performance and behaviour of chewing herbivores and their natural enemies and eventually the associated entire insect community?
2. Is there plant intraspecific variation in aphid-induced responses, and do they in turn differentially affect the subsequent attackers and alter the insect community as a whole?
3. Do the two major signal-transduction pathways underlying induced defences interact when plants are infested with both aphids and caterpillars either simultaneously or separated in time in different time sequences, and does this have consequences for direct and indirect plant defences?



## Study System

This thesis focused on plant interactions with multiple herbivores belonging to different feeding guilds, one phloem-feeding aphid species and various leaf-chewing herbivores species, as well as parasitoids of leaf chewers, using plants originating from wild cabbage populations.

### Plants

*Brassica oleracea* L. (Brassicaceae) is both an economically and ecologically important plant. The family of the Brassicaceae includes common agricultural crop species, like oilseed rape, radish, turnip, cabbage, kale, cauliflower, broccoli, etc. as well as the model plant species *Arabidopsis thaliana* (L.) Heynh. Brassicaceous plants characteristically produce glucosinolates, a group of well-studied plant secondary metabolites that are involved in plant direct and indirect defence against herbivore attack (Bukovinszky et al. 2005; Gols et al. 2008; Hopkins et al. 2009; Mumm and Dicke 2010). Upon tissue damage, glucosinolates are exposed to the enzyme myrosinase, resulting in the production of several breakdown compounds that may negatively affect a wide range of generalist herbivores (Hopkins et al. 2009). Specialist herbivores on brassicaceous plants are well-adapted to glucosinolates and have evolved specific detoxification and excretion strategies (Wittstock et al. 2004; Heckel 2014). Brassicaceous plants also release volatiles in response to herbivore attack, often referred to as herbivore-induced plant volatiles (HIPV), and these HIPV blends can provide reliable cues for host / prey location by natural enemies of insect herbivores (Dicke and Baldwin 2010; Hare 2011).

In the field, brassicaceous plants, both cultivated and wild host a complex insect community, including both generalist and specialist herbivores, phloem-feeding and leaf-chewing herbivores, as well as carnivorous insects at higher trophic levels (Moyes et al. 2000; Bukovinszky et al. 2008; Newton et al. 2009b; Poelman et al. 2013). Brassicaceous plants have been used to study tritrophic interactions between plants, herbivores and

parasitoids for years (Soler et al. 2005; Gols and Harvey 2008; Poelman et al. 2008; Gols et al. 2009). Moreover, ample information has been generated for brassicaceous plants on ecological, chemical and molecular aspects of their responses to single herbivory (Li et al. 2000; Moyes et al. 2000; Broekgaarden et al. 2007), and thus provides an excellent basis for the investigation of plant interactions with multiple herbivores. Brassicaceous plants exhibit considerable variation in direct and indirect defences against herbivory (Poelman et al. 2009; Gols et al. 2011), and as such also provide a good study system to address the research objectives of this thesis.



In this project, I used three wild *B. oleracea* populations that grow naturally along the south-coast of England. Seeds for my experiments had been collected in Dorset, U.K., at sites known as Kimmeridge (50°36'N, 2°07'W), Old Harry (OH; 50°38'N, 1°55'W), and Winspit (WIN; 50°35'N, 2°02'W), hereafter KIM, OH and WIN, respectively (Gols et al. 2008). Plants from these three wild cabbage populations display variation in both constitutive and herbivore-inducible foliar glucosinolates as well as variation in HIPV production (Mithen et al. 1995; Gols et al. 2008; Newton et al. 2009b).

### Herbivores

The specialist cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) mainly feeds on brassicaceous plants and sequesters glucosinolates from its food plants which in combination with endogenous myrosinases serve as a defence against their predators (Bridges et al. 2002; Kazana et al. 2007).

The diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae) is also a specialist herbivore on brassicaceous plant species and is considered the most destructive pest of *Brassica* crops (Furlong et al. 2013). *Plutella xylostella* larvae usually mine the leaf spongy mesophyll tissues during the first two larval developmental stages whereas as the last two larval stages feed on the abaxial surface of leaves, thereby often leaving the upper epidermis intact (Sarfraz et al. 2005).

*Pieris brassicae*, also known as the large cabbage white butterfly is also a specialist on plant species in the Brassicaceae family (Courtney and Chew 1987). Female butterflies of *P. brassicae* lay clutches of up to 150 eggs. Young larvae feed gregariously until they reach the fourth instar.

The cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae) is highly polyphagous, and feeds on more than 70 plant species from 22 families (Goulson and Cory 1995, Chougule et al. 2008). Eggs are laid in clutches of up to 200 eggs, but the caterpillars disperse immediately after egg hatching (Goulson and Cory 1995).

### Parasitoids

The larval endoparasitoid *Diadegma semiclausum* Helèn (Hymenoptera: Ichneumonidae) is considered a specialist parasitoid of *P. xylostella* hosts (Furlong et al. 2013). Females lay single eggs and can parasitize and successfully develop in all four larval stages of their host. Early larval stages of this parasitoid feed primarily on host haemolymph, whereas in the last stage of larval development all host tissues are consumed before the parasitoid pupates.

The larval endoparasitoid *Microplitis mediator* Haliday (Hymenoptera: Braconidae) can parasitize ca. 40 species of lepidopteran herbivores (Li et al. 2006). This solitary parasitoid can parasitize first-to-third larval instars of *M. brassicae* (Lauro et al. 2005). *Microplitis mediator* larvae feed exclusively on host haemolymph, and therefore only consume a small proportion of host tissues (Tanaka et al. 1984; Kim et al. 2008), before they egress from their host to pupate.

## Thesis outline

Plants are exposed to various herbivorous attackers in nature, and induced phenotypic changes in response to herbivory may affect the subsequently attacking herbivores. Chapter 2 presents a review of the recent progress in the study of plant responses to multiple herbivory and their effects on herbivores at different levels of biological complexity: from effects on the entire arthropod community to that on individual community members. In addition, mechanisms underlying the plant defensive responses to multiple herbivory were discussed with special attention to plant defence hormones and defence gene transcriptional responses to herbivory.

Chapter 3 addresses the effects of early-season aphid (*B. brassicae*) infestation on the composition and dynamics of the insect community associated with the three wild cabbage populations that vary in inducible and constitutive defence chemistry. Throughout the season, I monitored the insect community at different trophic levels and analysed the diversity and abundance of the detected insect species in response to early-season aphid infestation in a garden experiment. In addition, I investigated the effect of aphid infestation on parasitism of the common cabbage leaf-chewing herbivore *P. xylostella* by its parasitoids.

In Chapter 4, I determined the effect of initial aphid infestation on the performance of the diamondback moth *P. xylostella* and the cabbage moth *M. brassicae* and their respective parasitoids *D. semiclausum* and *M. mediator*. In particular, I investigated whether plants of the three cabbage populations exhibit intraspecific variation in their response to aphid infestation, and whether these differentially affect the performance of the two leaf-chewing herbivores and their endoparasitoids.

To investigate whether co-infestation with phloem-feeding aphids interferes with volatile-mediated foraging behaviour of parasitoids attacking chewing caterpillars, I studied the effect of aphid infestation and its duration on indirect plant responses against leaf-chewing herbivores in the three wild cabbage populations (Chapter 5). In two-choice bioassays, I investigated the relative attractiveness of plant volatiles emitted by a host-infested (either by caterpillars of *P. xylostella* or *M. brassicae*) and a dually infested plant (hosts plus aphids) to their respective parasitoids *D. semiclausum* and *M. mediator*. I also investigated whether differences in volatile chemistry could explain preference behaviour of the two parasitoids.



Antagonism between SA and JA defence signalling may help plant to fine-tune their responses to different attackers in time, but also potentially constrain a plant's ability to simultaneously defend itself against multiple attackers that activate different signal transduction pathways. In Chapter 6, I studied the effect of the temporal sequence of infestation by the aphid *B. brassicae* and three caterpillar species, *P. xylostella*, *P. brassicae*, and *M. brassicae,* on plant activation of JA and SA signal-transduction pathways in three wild cabbage populations by quantifying the transcript levels of an SA- and a JA-responsive marker gene.

Finally, in Chapter 7, the findings of this thesis are discussed with an emphasis on the integration of the results obtained at different levels of biological organisation. I discuss how feeding guild determines the specificity of the herbivore-induced plant response, and in turn how this affects subsequent herbivores at the level of the individual, but also at the level of the entire associated insect community. I focus on plant hormonal signalling and the emission of plant volatiles as induced by chewing herbivores and how simultaneous aphid infestation affects their induction, as well as direct and indirect plant defences against leaf-chewing herbivores. Finally, I discuss future directions of the research on plant responses to multiple herbivory by insects belonging to different feeding guilds. The research on plant response to multiple herbivory can yield practical applications for improving plant resistance and agricultural productivity in the future.

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## References


Agrawal AA (1999a) Induced response to herbivory in wild radish: effects on several herbivores and plant fitness. Ecology 80: 1713-1723.

Agrawal AA, Hastings AP, Patrick ET, Knight AC (2014) Specificity of herbivore-induced hormonal signaling and defensive traits in five closely related milkweeds (*Asclepias* spp.). Journal of Chemical Ecology 40: 717-729.

Agrawal AA (1999) Induced plant defense: evolution of induction and adaptive phenotypic plasticity. In Agrawal AA, Tuzun S, Bent E (Eds.) *Induced Plant Defenses Against Pathogens and Herbivores. Biochemstry, Ecology and Agriculture* (pp. 251-268). St. Paul, Minosota: APS Press.

Agrawal AA (2000) Specificity of induced resistance in wild radish: causes and consequences for two specialist and two generalist caterpillars. Oikos 89: 493-500.

Agrawal AA, Hastings AP, Johnson MT, Maron JL, Salminen JP (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. Science 338: 113-116.

Agrawal AA, Karban R (1999) Why induced defenses may be favored over constitutive strategies in plants. In R. Tollrian and C. D. Harvell (Eds.), *The ecology and evolution of inducible defenses* (PP. 45-61). Princeton, US: Princeton University Press.

Bridges M, Jones AM, Bones AM, Hodgson C, Cole R, Bartlet E, Wallsgrove R, Karapapa VK, Watts N, Rossiter JT (2002) Spatial organization of the glucosinolate-myrosinase system in brassica specialist aphids is similar to that of the host plant. Proceedings of the Royal Society B: Biological Sciences 269: 187–191.

Broekgaarden C, Poelman EH, Steenhuis G, Voorrips RE, 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.

Bukovinszky T, Gols R, Kamp A, de Oliveira-Domingues F, Hamback PA, Jongema Y, Bezemer TM, Dicke M, van Dam NM, Harvey JA (2010) Combined effects of patch size and plant nutritional quality on local densities of insect herbivores. Basic and Applied Ecology 11: 396-405.

Bukovinszky T, Gols R, Posthumus MA, Vet LEM, van Lenteren JC (2005) Variation in plant volatiles and attraction of the parasitoid *Diadegma semiclausum* (Hellen). Journal of Chemical Ecology 31: 461-480.

Bukovinszky T, van Veen FJF, Jongema Y, Dicke M (2008) Direct and indirect effects of resource quality on food web structure. Science 319: 804-807.

Chougule NP, Doyle E, Fitches E, Gatehouse JA (2008) Biochemical characterization of midgut digestive proteases from *Mamestra brassicae* (cabbage moth; Lepidoptera: Noctuidae) and effect of soybean Kunitz inhibitor (SKTI) in feeding assays. Journal of Insect Physiology 54: 563-572.

Courtney S, Chew F (1987) Coexistence and host use by a large community of Pierid butterflies: habitat is the templet. Oecologia 71: 210-220.

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Métraux JP, Van Loon JC, Dicke M, Pieterse CMJ (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. Molecular Plant-Microbe Interactions 18: 923-937.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends in Plant Science 15: 167-175.

Dungey HS, Potts BM, Whitham TG, Li HF (2000) Plant genetics affects arthropod community richness and composition: evidence from a synthetic eucalypt hybrid population. Evolution 54: 1938-1946.

Erb M, Meldau S, Howe GA (2012) Role of phytohormones in insect-specific plant reactions. Trends in Plant Science 17: 250-259.

Furlong MJ, Wright DJ, Dosdall LM (2013) Diamondback moth ecology and management: problems, progress, and prospects. Annual Review of Entomology 58: 517-541.

Gols R, Bullock JM, Dicke M, Bukovinszky T, Harvey JA (2011) Smelling the wood from the trees: non-linear parasitoid responses to volatile attractants produced by wild and cultivated cabbage. Journal of Chemical Ecology 37: 795-807.

Gols R, Harvey JA (2008) Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. Phytochemistry Reviews 8: 187-206.

Gols R, van Dam NM, Raaijmakers CE, Dicke M, Harvey JA (2009) Are population differences in plant quality reflected in the preference and performance of two endoparasitoid wasps? Oikos 118: 733-743.

Gols R, Wagenaar R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM, Harvey JA (2008) Genetic variation in defense chemistry in wild cabbages affects herbivores and their endoparasitoids. Ecology 89: 1616-1626.

Goulson D, Cory JS (1995) Responses of *Mamestra brassicae* (Lepidoptera: Noctuidae) to crowding: interactions with disease resistance, colour phase and growth. Oecologia 104: 416-423.

Hare JD (2011) Ecological role of volatiles produced by plants in response to damage by herbivorous insects. Annual Review of Entomology 56: 161-180.

Heckel DG (2014) Insect detoxification and sequestration strategies. In C. Voelckel and G. Jander (Eds.), *Annual Plant Reviews volume 47: Insect-Plant Interactions* (pp.77-114). Oxford, UK: John Wiley & Sons, Ltd.

Hopkins RJ, van Dam NM, Van Loon JJA (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annual Review of Entomology 54: 57-83.

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annual Review of Plant Biology 59: 41-66.

Johnson MT, Agrawal AA, Maron JL, Salminen J (2009) Heritability, covariation and natural selection on 24 traits of common evening primrose (*Oenothera biennis*) from a field experiment. Journal of Evolutionary Biology 22: 1295-1307.

Johnson MTJ, Lajeunesse MJ, Agrawal AA (2006) Additive and interactive effects of plant genotypic diversity on arthropod communities and plant fitness. Ecology Letters 9: 24-34.

Karban R, Baldwin IT (1997) Induced responses to herbivory. University of Chicago Press. Chicago, Illinois, USA.

Kazana E, Pope TW, Tibbles L, Bridges M, Pickett JA, Bones AM, Powell G, Rossiter JT (2007) The cabbage aphid: a walking mustard oil bomb. Proceedings of the Royal Society B: Biological Sciences 274: 2271-2277.

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annual Review of Plant Biology 53: 299-328.

Kessler A, Baldwin IT (2004) Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco *Nicotiana attenuata*. The Plant Journal 38: 639-649.

Kessler A, Halitschke R (2007) Specificity and complexity: the impact of herbivore-induced plant responses on arthropod community structure. Current Opinion in Plant Biology 10: 409-414.

Kim YH, Kang JS, Kim JI, Kwon M, Lee S, Cho HS, Lee SH (2008) Effects of Bt transgenic Chinese cabbage on the herbivore *Mamestra brassicae* (Lepidoptera: Noctuidae) and its parasitoid *Microplitis mediator* (Hymenoptera: Braconidae). Journal of Economic Entomology 101: 1134-1139.

Kliebenstein DJ, Kroymann J, Brown P, Figuth A, Pedersen D, Gershenzon J, Mitchell-Olds T (2001) Genetic control of natural variation in Arabidopsis glucosinolate accumulation. Plant Physiology 126: 811-825.

Lauro N, Kuhlmann U, Mason P, Holliday N (2005) Interaction of a solitary larval endoparasitoid, *Microplitis mediator*, with its host, *Mamestra brassicae*: host acceptance and host suitability. Journal of Applied Entomology 129: 567-573.

Li J, Yan F, Coudron TA, Pan W, Zhang X, Liu X, Zhang Q (2006) Field release of the parasitoid *Microplitis mediator* (Hymenoptera: Braconidae) for control of *Helicoverpa armigera* (Lepidoptera: Noctuidae) in cotton fields in northwestern China's Xinjiang province. Environmental Entomology 35: 694-699.

Li Q, Eigenbrode SD, Stringham GR, Thiagarajah MR (2000) Feeding and growth of *Plutella xylostella* and *Spodoptera eridania* on *Brassica juncea* with varying glucosinolate concentrations and myrosinase activities. Journal of Chemical Ecology 26: 2401-2419.

Lou Y, Hua X, Turlings TJ, Cheng J, Chen X, Ye G (2006) Differences in induced volatile emissions among rice varieties result in differential attraction and parasitism of *Nilaparvata lugens* eggs by the parasitoid *Anagrus nilaparvatae* in the field. Journal of Chemical Ecology 32: 2375-2387.

Mewis I, Appel HM, Hom A, Raina R, Schultz JC (2005) Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiology 138: 1149-1162.

Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C, Gershenzon J (2006) Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. Phytochemistry 67: 2450-2462.

Mithen R, Raybould AF, Giamoustaris A (1995) Divergent selection for secondary metabolites between wild populations of *Brassica oleracea* and its implications for plant-herbivore interactions. Heredity 75: 472-484.

Moyes CL, Collin HA, Britton G, Raybould AE (2000) Glucosinolates and differential herbivory in wild populations of *Brassica oleracea*. Journal of Chemical Ecology 26: 2625-2641.

Mumm R, Dicke M (2010) Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. Canadian Journal of Zoology 88: 628-667.

Newton EL, Bullock JM, Hodgson DJ (2009a) Bottom-up effects of glucosinolate variation on aphid colony dynamics in wild cabbage populations. Ecological Entomology 34: 614-623.

Newton EL, Bullock JM, Hodgson DJ (2009b) Glucosinolate polymorphism in wild cabbage (*Brassica oleracea*) influences the structure of herbivore communities. Oecologia 160: 63-76.

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nature Chemical Biology 5: 308-316.

Poelman EH, Broekgaarden C, Van Loon JJA, 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 EH, Dam NM, Loon JJA, Vet LEM, Dicke M (2009) Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores. Ecology 90: 1863-1877.

Poelman EH, Harvey JA, Van Loon JJA, Vet LEM, Dicke M (2013) Variation in herbivore-induced plant volatiles corresponds with spatial heterogeneity in the level of parasitoid competition and parasitoid exposure to hyperparasitism. Functional Ecology 27: 1107-1116.

Rodriguez-Saona CR, Musser RO, Vogel H, Hum-Musser SM, Thaler JS (2010) Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. Journal of Chemical Ecology 36: 1043-1057.

Sarfraz M, Keddie AB, Dosdall LM (2005) Biological control of the diamondback moth, *Plutella xylostella*: A review. Biocontrol Science and Technology 15 (8): 763-789.

Schmidt DD, Voelckel C, Hartl M, Schmidt S, Baldwin IT (2005) Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. Plant Physiology 138: 1763-1773.

Schoonhoven LM, Van Loon JJA, Dicke M (2005) Insect-Plant Biology, 2nd edn. Oxford University Press, Oxford, UK.

Sole R, Bezemer T, Van Der Putten WH, Vet LEM, Harvey JA (2005) Root herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid performance via changes in plant quality. Journal of Animal Ecology 74: 1121-1130.

Stam JM, Kroes A, Li Y, Gols R, Van Loon JJA, Poelman EH, Dicke M (2014) Plant interactions with multiple insect herbivores: from community to genes. Annual Review of Plant Biology 65: 689-713.

Tanaka T, Sato Y, Hidaka T (1984) Developmental interaction between *Leucania separate* (Lepidoptera: Noctuidae) and its Braconid parasitoid, *Microplitis mediator* (Hymenoptera: Braconidae). Journal of Economic Entomology 77: 91-97.

Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science 17: 260-270.

Van Zandt PA, Agrawal AA (2004a) Community-wide impacts of herbivore-induced plant reaponses in milkweed (*Asclepias syriaca*). Ecology 85: 2616-2629.

Van Zandt PA, Agrawal AA (2004b) Specificity of induced plant responses to specialist herbivores of the common milkweed *Asclepias syriaca*. Oikos 104: 401-409.

Viswanathan DV, Narwani AJT, Thaler JS (2005) Specificity in induced plant responses shapes patterns of herbivore occurrence on *Solanum dulcamara*. Ecology 86: 886-896.

Voelckel C, Baldwin IT (2004) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. The Plant Journal 38: 650-663.

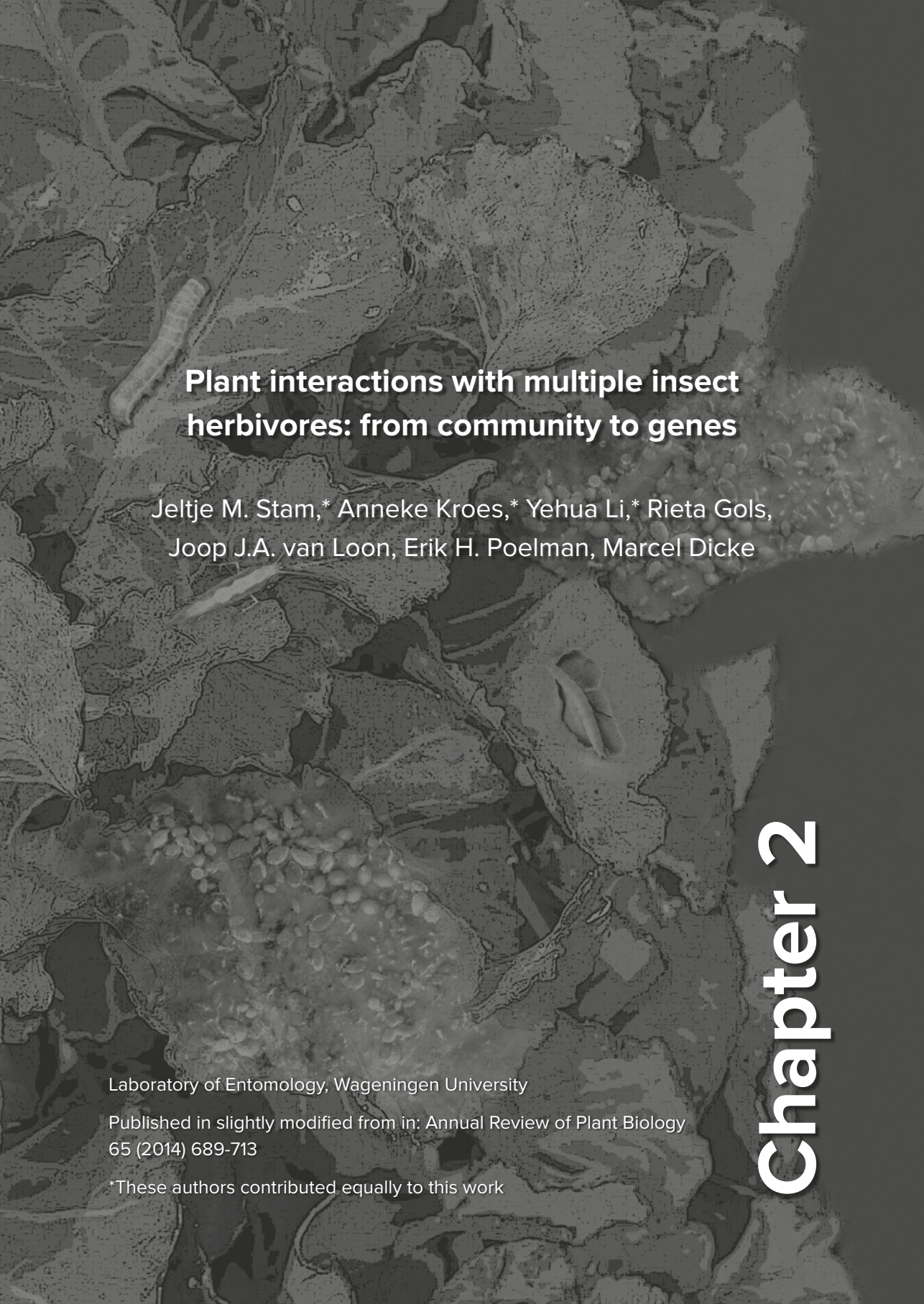
Wittstock U, Agerbirk N, Stauber EJ, Olsen CE, Hippler M, Mitchell-Olds T, Gershenzon J, Vogel H (2004) Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proceedings of the National Academy of Sciences of the United States of America 101: 4859-4864.

Wu J, Baldwin IT (2009) Herbivory-induced signalling in plants: perception and action. Plant, Cell and Environment 32: 1161-1174.

Wu J, Baldwin IT (2010) New insights into plant responses to the attack from insect herbivores. Annual Review of Genetics 44: 1-24.

Wu J, Hettenhausen C, Schuman MC, Baldwin IT (2008) A comparison of two *Nicotiana attenuata* accessions reveals large differences in signaling induced by oral secretions of the specialist herbivore *Manduca sexta*. Plant Physiology 146: 927-939.

Zhang P, Broekgaarden C, Zheng SJ, Snoeren TAL, Van Loon JJA, Gols R, Dicke M (2013) Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. New Phytologist 197: 1291-1299.



# Plant interactions with multiple insect herbivores: from community to genes

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## Abstract

Every plant is a member of a complex insect community that consists of tens to hundreds of species that belong to different trophic levels. The dynamics of this community are critically influenced by the plant, which mediates interactions between community members that can occur on the plant simultaneously or at different times. Herbivory results in changes in the plant's morphological or chemical phenotype that affect interactions with subsequently arriving herbivores. Changes in the plant's phenotype are mediated by molecular processes such as phytohormonal signaling networks and transcriptomic rearrangements that are initiated by oral secretions of the herbivore. Processes at different levels of biological complexity occur at timescales ranging from minutes to years. In this review, we address plant-mediated interactions with multiple species of the associated insect community and their effects on community dynamics, and link these to the mechanistic effects that multiple attacks have on plant phenotypes.

**Keywords:** Phenotypic plasticity, trait-mediated interaction networks, phytohormones, systems biology, species interactions

Plants are members of biodiverse communities consisting of a microbiome (Mendes et al., 2011) and a macrobiome (Dicke and Baldwin, 2010; Whitham et al., 2010). The microbiome consists of, e.g., symbiotic microorganisms such as mycorrhizae, endophytes, and nitrogen-fixing bacteria; plant pathogenic microorganisms; and their antagonists (Hartley and Gange, 2009; Pineda et al., 2010). The macrobiome consists of herbivores and their natural enemies, such as predators and parasitoids, as well as pollinators (Fig 1). For each plant species, the combined macrobiome and microbiome can easily comprise several hundred species that belong to different trophic levels (Harvey et al., 2009) (Fig 1). Moreover, each individual plant is surrounded by a range of other plant individuals of the same or different species, which compete for light and nutrients (Cerrudo et al., 2012) and share members of the microbiome and macrobiome.



Understanding the functioning of this complex of interacting species requires studies of their population dynamics in space and time and the underlying trophic and informational mechanisms. In this review, we focus on plants and their associated insect communities. Insects are the most speciose group of organisms, comprising an estimated 6 million species, of which 50% are herbivorous, and the 300,000 plant species represent the group of organisms with the largest biomass (Schoonhoven et al., 2005). Thus, communities of insects and plants make up a significant proportion of life on Earth.

Feeding by herbivorous insects influences the phenotype of their food plant (Dicke and Baldwin, 2010; Kessler and Baldwin, 2002; Mithöfer and Boland, 2012), which consequently influences the interactions of the plant with its associated community (Ohgushi, 2008; Poelman et al., 2011; Utsumi et al., 2010). Such herbivore-induced effects may last throughout the growing season of the plant or for several years (Haukioja, 1980; Johnson and Agrawal, 2007; Poelman et al., 2008; Thaler et al., 2001).

Research on plant–insect interactions has addressed mainly the effects of interactions between one plant and one insect species. This has yielded important knowledge on how insects find and select their host plants and deal with plant defenses (Schoonhoven et al., 2005) as well as how herbivory modifies plant phenotypes (Dicke and Baldwin, 2010; Kessler and Baldwin, 2002; Mithöfer and Boland, 2012). However, because plants are members of complex communities, interactions with multiple attackers are the rule rather than an exception (Dicke et al., 2009; Ohgushi, 2005; Utsumi et al., 2010). Moreover, attacks by different organisms interact at different levels of biological organization, ranging from the subcellular level (Pieterse et al., 2012) to the individual (Kaplan and Denno, 2007) and community levels (Poelman et al., 2009). Studies on the interactions between plants and their associated insect communities have received increasing attention and have addressed effects at the levels of gene expression, phytohormonal crosstalk, metabolomic changes, species interactions, and community dynamics. The current focus in the field of plant–insect interactions is on connecting different levels of biological organization (Baldwin, 2012; Keurentjes et al., 2011), which is already challenging for individual plant–

insect interactions and certainly so for multiple attacks on a single plant, and therefore requires a multidisciplinary approach.

In this review, we address the effects of multiple attacks on plants at different levels of biological organization in an integrative way. Although plants are members of plant communities that comprise individuals from different species, we limit this review to individual plants, and particularly interactions with multiple insect species aboveground. We also limit the review to plants in the vegetative stage, because most information is available for this plant stage. We conclude with an outlook on the future of this rapidly developing, multidisciplinary field.



## Plants affect insect community composition and dynamics

The composition and dynamics of the insect community that interacts with a plant are influenced by plant traits such as chemistry, physiology, and morphology (Bukovinszky et al., 2008: Harvey et al., 2011; Johnson et al., 2006; Ohgushi, 2005; Whitham et al., 2006), which have a genetic basis. Thus, the genotype of a plant and, consequently, the expressed plant phenotype affect insect community members that interact with the plant and shape the composition of the community (Whitham et al., 2006; Whitham et al., 2012). The insect community together with the plant phenotype gives rise to the community phenotype, and plant individuals with similar traits tend to support similar insect communities (Johnson et al., 2006; Keith et al., 2010; Whitham et al., 2006). A plant's genotype can have size- and density-mediated effects on the associated insect community. For example, plant traits may affect the sizes of herbivores and therefore the sizes of parasitoids (Fig 1D,E) that develop in the herbivores, and even the sizes of hyperparasitoids (Fig 1F,G) that develop in those parasitoids that develop in the herbivores (Bukovinszky et al., 2008). Moreover, plant genotype may affect the density of herbivores, parasitoids, and hyperparasitoids as well as the composition of the herbivore, parasitoid, and hyperparasitoid communities on these plants (Bukovinszky et al., 2008).

Chemical plant traits are well known to be crucial components of the plant phenotype that mediate plant–insect interactions (Schoonhoven et al., 2005). Genotypic variation affects plant chemical traits, which has consequences for species interactions and community dynamics. An example of an extensively studied plant chemical trait that affects insect community composition is condensed tannin concentration, especially in tree species (Schweitzer et al., 2008; Whitham et al., 2006). Tannins are known to negatively influence herbivorous insects (Schoonhoven et al., 2005), and the concentration of tannins in poplar trees indeed affects the composition of insect communities (Whitham et al., 2006). Tannins usually reduce insect growth rate (Schoonhoven et al., 2005), although tannins may also positively affect insect performance or preference; the effects of tannins are likely dependent on species, tissue, and context and influenced by other chemical constituents

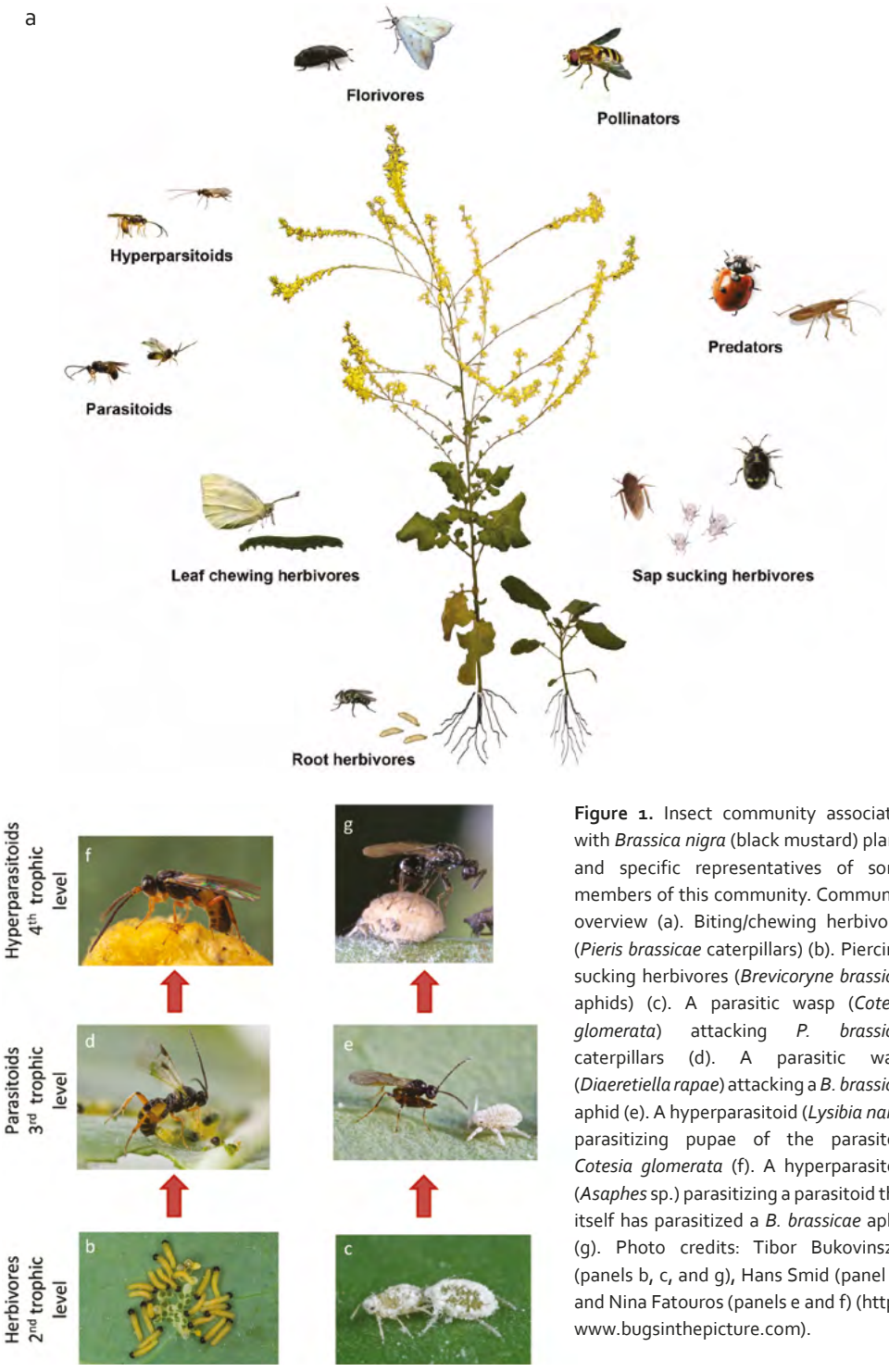


**Figure 1.** Insect community associated with *Brassica nigra* (black mustard) plants and specific representatives of some members of this community. Community overview (a). Biting/chewing herbivores (*Pieris brassicae* caterpillars) (b). Piercing/sucking herbivores (*Brevicoryne brassicae* aphids) (c). A parasitic wasp (*Cotesia glomerata*) attacking *P. brassicae* caterpillars (d). A parasitic wasp (*Diaeretiella rapae*) attacking a *B. brassicae* aphid (e). A hyperparasitoid (*Lysibia nana*) parasitizing pupae of the parasitoid *Cotesia glomerata* (f). A hyperparasitoid (*Asaphes* sp.) parasitizing a parasitoid that itself has parasitized a *B. brassicae* aphid (g). Photo credits: Tibor Bukovinszky (panels b, c, and g), Hans Smid (panel d), and Nina Fatouros (panels e and f) (http://www.bugsinthepicture.com).

of plant tissue (Schweitzer et al., 2008). Tannins can also affect community members indirectly through a negative effect on nitrogen mineralization, which subsequently feeds back to root production and consequently to the nutritional value of the tree (Whitham et al., 2006), with long-term effects on herbivorous insects (Schweitzer et al., 2008). Thus, condensed tannin levels affect community phenotypes (Whitham et al., 2006).

In annual or perennial nonwoody plant species, family-specific secondary chemistry can shape the community phenotype. For instance, glucosinolates, which are characteristic secondary metabolites of plants in the Brassicaceae family, have important effects on insect community composition (Hopkins et al., 2009; Newton et al., 2009a; Poelman et al., 2009). The quality and quantity of these compounds are known to deter generalist insect species or hamper their development, whereas they may be used for feeding and or as oviposition stimulants by specialist species (Hopkins et al., 2009). Differences in glucosinolate composition among *Brassica oleracea* cultivars resulted in large differences in herbivore community dynamics (Poelman et al., 2009) that resemble community differences observed in natural populations of *B. oleracea* plants that differ in their chemical profiles (Newton et al., 2009a).



In addition to plant secondary chemistry, many other plant traits can affect insects. These traits include plant biomass and architecture (Andow, 1991; Johnson and Agrawal, 2005; Ohgushi, 2005; Schoonhoven et al., 2005), leaf morphology (Barbour et al., 2009), trichome density (Johnson, 2008), and plant nutritional value in terms of water and nitrogen content (Johnson, 2008; Scriber and Slansky, 1981).

### Consequences of plant traits for insect herbivores

To understand how a plant's genotype affects community composition and dynamics, knowledge of the underlying mechanisms is important. Individual plant traits have different effects on different community members. Among insect herbivores contrasting dietary categories are observed. Generalist species feed on plants belonging to phytochemically unrelated families, whereas specialist species utilize only plant species within a single family or a single genus (Ali and Agrawal, 2012; Schoonhoven et al., 2005). Generalist herbivores are usually more sensitive to plant defense compounds, whereas specialist herbivores may use these same compounds as recognition cues (known as token stimuli) (Gols et al., 2008a; Gols et al., 2008b). Adaptation to plant chemicals specific for certain plant taxa through specialized detoxification or sequestration mechanisms allows specialists to utilize some plants as food and exploit such chemicals for their own defense, whereas generalists are either unable to survive or grow or have a reduced survival or growth rate on such plants (Ali and Agrawal, 2012; Hopkins et al., 2009).

Insect herbivores can also be classified based on feeding guilds—e.g., leaf chewers, phloem feeders, leaf miners, root feeders, and gall-inducing insects—which may differ in their responses to plant traits. Whereas leaf chewers often consume whole leaves and thus are exposed to chemicals in all leaf cells, phloem feeders such as aphids specialize on the phloem. Some secondary compounds that react with each other to form a toxic compound only upon rupture of multiple cells by chewing are thus circumvented by piercing/sucking phloem feeders (Schoonhoven et al., 2005).

Some plant traits are likely to affect all herbivores, whereas others affect only a particular subset, e.g., based on herbivore size. A plant with high leaf toughness will affect many herbivore species, although some species are better able to deal with this than others (Agrawal, 2005). In contrast, a high trichome density particularly affects smaller insects that walk in a forest of leaf hairs (Dussourd, 1995; Schoonhoven et al., 2005), and secondary metabolites particularly affect generalist insects (see above).



Because distinct herbivorous members of a community respond differently to the same plant traits, each trait differentially influences community composition. The many interactions that occur between the various plant traits and the diverse community members, and among herbivore members themselves, potentially increase the complexity of underlying mechanisms that modulate community composition. However, only one or a few so-called foundation species may have a major effect on the community composition (Whitham et al., 2006). Keith et al. (2010) proposed that a few plant traits particularly affect one or a few foundation herbivore species, which subsequently affect the community. This suggests that effects of plant traits might be passed on not only to single species but also to a whole chain of interacting species.

### Consequences of plant traits for insect carnivores

The discussion above considered mainly plant–herbivore interactions, but plant traits also affect organisms at higher trophic levels, such as predators or parasitoids of herbivores as well as carnivorous insects at even higher trophic levels (Bukovinszky et al., 2008; Dicke and Baldwin, 2010; Harvey et al., 2009; Heil, 2008; McCormick et al., 2012; Poelman et al., 2012; Price et al., 1980). Plant traits can directly affect the natural enemies of herbivores, for example, by providing shelter (Romero and Benson, 2005; Schoonhoven et al., 2005) or extrafloral nectar as food (Heil et al., 2010; Schoonhoven et al., 2005). Plant traits can also affect higher trophic levels either directly, through reduced quality of the herbivores (Bukovinszky et al., 2008), or indirectly, through exposure to phytochemicals ingested by the herbivore (Gols and Harvey, 2009). Such indirect interactions with herbivores as a mediator between plant traits and predators or parasitoids can have large effects on the community composition at the second, third, and even higher trophic levels (Bukovinszky et al., 2008; Harvey et al., 2009; Poelman et al., 2012; Smith et al., 2011; Whitham et al., 2006).

For example, evening primrose genotype affected aphid population growth rate directly as well as indirectly through effects on the abundance of aphid-tending ants and the diversity of predators (Johnson, 2008). Similar results were found for parasitoids of caterpillars feeding on genetically different willows (Fritz et al., 1997) or cabbage plants (Bukovinszky et al., 2008; Harvey et al., 2011). The adaptation of herbivores to specific plant traits might even affect the evolution of members of higher trophic levels, leading to specialization of parasitoids on herbivores that are adapted to plant traits (Stireman et al., 2006). Plant effects on the composition of the herbivore community can also affect the foraging behavior of carnivores. For example, the foraging success of parasitoids that search for hosts is affected by the presence and identity of additional, nonhost herbivores on the plant (De Rijk et al., 2013).



Plant traits may also interfere with the performance of carnivorous insects, thereby providing herbivores with enemy-free space. For instance, in pea plants, a leafless mutation that affects plant architecture hampers the foraging behavior of lady beetles, which results in enhanced population growth of aphids (Kareiva and Sahakian, 1990).

### Top-down effects

The bottom-up effects of plant traits on higher trophic levels (herbivores and their natural enemies) discussed above may be strong predictors of community composition (Kos et al., 2011), but top-down effects of natural enemies on herbivores can have important effects on community composition as well (Hunter and Price, 1992). Predators or parasitoids consume their hosts partly or completely and therefore constrain the population density of herbivores attacking a plant. Parasitoids can exert significant top-down control of herbivore populations (Van Veen et al., 2005), and their activities can influence competition between herbivore species (Van Veen et al., 2006). Interestingly, the elimination of a single parasitoid species from a small community resulted in the extinction of other parasitoid species that were four trophic links away (Sanders et al., 2013). This included effects mediated through herbivores. Thus, top-down effects can be sequentially linked to bottom-up effects (Kareiva and Sahakian, 1990; Sanders et al., 2013).

Insects at the third trophic level do not always have a negative effect on herbivore species: For example, ants may tend aphids and thus protect them from their natural enemies (Johnson, 2008). Although the ants have a positive effect on the aphids, they may also prey on other herbivores that share the plant with the aphids (Vrieling et al., 1991).

In conclusion, plant traits influence members of the associated insect community at different trophic levels, and species at higher trophic levels affect the dynamics of species at lower trophic levels. Many of these plant traits are constitutively expressed. Moreover, community dynamics are also influenced in important ways by the fact that insects modify plant phenotype. The modification of plant phenotype by herbivore attack is the focus of the remainder of this article. We address the effects of phenotypic modification by herbivory

on insects at different trophic levels, the molecular mechanisms underlying the phenotypic modification, and how different herbivore species that attack the same plant interfere with one another's effects on the plant's phenotype. Finally, we address the effects of herbivore-induced modification of plant phenotype on community dynamics.

## Herbivore-induced change in plant traits

Herbivory or egg deposition by herbivores alters plant phenotype through changes in the production of primary and secondary metabolites, morphological traits, and architecture (Dicke and Baldwin, 2010; Hilker and Meiners, 2010; Howe and Jander, 2008; Kessler and Baldwin, 2002; Mithöfer and Boland, 2012) (Fig 2). Such herbivore-induced plant responses may affect the behavior and growth of the initial attacker and may also influence host-plant suitability for other herbivores, even when these are temporally or spatially separated, thus mediating interspecific competition between insect herbivores (Denno et al., 1995; Ohgushi, 2005; Ohgushi, 2008) (Fig 2). Furthermore, the effects of herbivore-induced alterations in plant phenotype are to some extent specific to the attacking herbivores, and they may affect subsequent herbivores either positively or negatively, depending on the characteristics of the responding herbivore species (Kaplan and Denno, 2007). For example, spider-mite infestation of cotton plants increased resistance against conspecific mites and whiteflies but also enhanced susceptibility to aphids (Agrawal et al., 2000). Willow infestation by leaf rollers enhanced the abundance of aphids and ants but also reduced the abundance of leaf beetles (Ohgushi, 2005).

### Herbivore-induced resistance to herbivores

Herbivore-induced resistance of plants to herbivores is a common phenomenon and has been described for many insect herbivores of various feeding guilds (Karban and Baldwin, 1997). For example, through induced changes in plant phenotype, feeding by lepidopteran larvae prolongs immature development of other lepidopteran species that colonize a common host plant later in the season (Agrawal, 2000; Poelman et al., 2008).

A meta-analysis of genetic correlations between plant levels of resistance to multiple enemies revealed positive correlations when the compared species were both generalist herbivores or when they were both specialist herbivores (Leimu and Koricheva, 2006). It also revealed significant positive genetic correlations for plant resistance to herbivores from different feeding guilds, such as miners and gall inducers, miners and leaf folders, and gall inducers and leaf folders (Leimu and Koricheva, 2006). In pairwise comparisons of interactions between herbivores belonging to different feeding guilds, the lowest genetic correlation was recorded for mechanisms of plant resistance to phloem-feeding and leaf-chewing herbivores (Leimu and Koricheva, 2006).

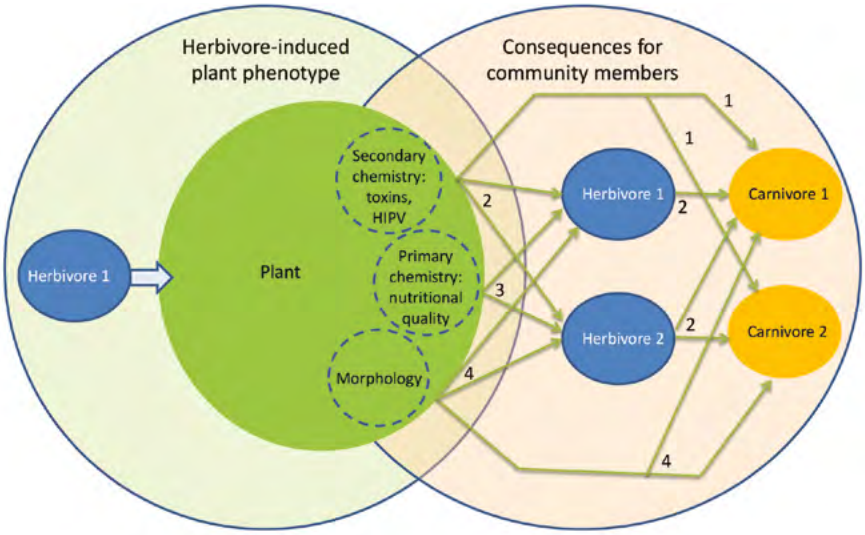


**Figure 2.** Components of plant phenotypic plasticity in response to herbivore attack and the interactions of these components with other members of the insect community: ①, herbivore-induced plant volatiles that attract carnivorous insects (HIPV); ②, secondary plant metabolites such as toxins and digestibility reducers that affect the performance of herbivores and through herbivores may affect their carnivorous enemies; ③, primary plant metabolites that are used as nutrients by herbivores; ④, morphological characteristics such as trichomes and cuticular wax layers that affect the performance of herbivorous insects and the behavior of their carnivorous enemies.

## Herbivore-induced susceptibility to herbivores

Herbivore-induced susceptibility seems to be less common than herbivore-induced resistance (Leimu and Koricheva, 2006), and in half of the reported cases it involved interactions between piercing/sucking and biting/chewing herbivores (Denno et al., 1995). Yet 20–40% of the total number of interactions within the herbivore community associated with willow and goldenrod were facilitative (Ohgushi, 2008). Most facilitative interactions were asymmetric, with only one species gaining an advantage (Denno et al., 1995; Kaplan and Denno, 2007).

Different mechanisms may underlie facilitation among different herbivore species. For example, the facilitative interaction between spittlebugs and leaf rollers that was observed on willow was caused by compensatory shoot growth in response to spittlebug infestation; leaf rollers prefer leaves on the new shoots (Ohgushi, 2005). A stem-boring moth induced susceptibility in willow to a specialist leaf beetle by causing young shoot growth (Utsumi and Ohgushi, 2008). Herbivory by leaf rollers on oak provided shelter and better feeding sites for aphids (Karban and Agrawal, 2002; Karban et al., 1997), and herbivory by aphids interfered with induced defense signaling against caterpillars (Soler et al., 2012).

### Herbivore-induced plant responses and carnivorous insects

Herbivore-induced changes in plant secondary chemistry play an important role in habitat and host location of carnivorous insects, mainly via the production of volatiles in response to feeding by their prey or hosts (D'Alessandro and Trulings, 2006; Dicke and Baldwin, 2010; McCormick et al., 2012) (Fig 2). These herbivore-induced plant volatiles attract the carnivorous enemies of herbivores to plants infested with their herbivorous victim. Moreover, even hyperparasitoids at the fourth trophic level may exploit herbivore-induced plant volatiles to find their parasitoid host that feeds within an herbivorous insect (Poelman et al., 2012). However, specific volatile chemicals or mixtures of chemicals may also repel carnivorous insects (Braasch et al., 2012; Snoeren et al., 2010; Webster et al., 2010). Volatile-mediated foraging behavior of carnivores is more difficult to predict when multiple herbivores attack the same host plant (Dicke et al., 2009; Ponzio et al., 2013; Shiojiri et al., 2001). When nonhost herbivores share the same plant individual with hosts, changes in the induced volatile blend can interfere with host location by foraging carnivorous insects (De Rijk et al., 2013; Dicke et al., 2009).

Nonvolatile plant chemistry may also mediate the effects of herbivore-induced changes in plant phenotype on carnivores (reviewed in Gols and Harvey (2009) and Ode (2006)). Herbivore-induced changes in plant chemistry may prolong herbivore development and consequently extend the exposure period of the herbivore to its enemies (Benrey and Denno, 1997). Moreover, some specialist herbivores are able to sequester plant secondary metabolites and exploit these defenses for their own protection from natural enemies (Kazana et al., 2007; Müller, 2009) (Fig 2). Herbivore-induced plants may also influence immune responses of herbivores to parasitoids (Bukovinszky et al., 2009). *Pieris rapae* caterpillars that developed on plants previously damaged by *Pieris brassicae* caterpillars had a reduced ability to encapsulate parasitoid eggs compared with those reared on undamaged plants (Bukovinszky et al., 2009). It is remarkable that herbivory resulted in inferior performance and immune response of the subsequent caterpillars and enhanced their susceptibility to parasitism. However, suppressed performance of host caterpillars on induced plants may also inhibit parasitoid performance through reduced host nutrient availability (Ode, 2006). Generalist parasitoids tend to be more susceptible to inducible plant metabolites than specialist parasitoids are (Bukovinszky et al., 2012; Gols et al., 2008b).

In conclusion, herbivory alters plant phenotype, which has consequences for the interactions of the plant with herbivorous and carnivorous insects (Fig 2). In the next section, we address the molecular mechanisms underlying the modification of plant phenotype by herbivory and how different herbivores feeding on the same plant affect one another's modifications.

## Molecular mechanisms underlying plant phenotypic plasticity under single and multiple attacks

The past decade has brought significant advances in the mechanistic understanding at the (sub)cellular level of induced plant responses that underlie plant–insect interactions (Bonaventure et al., 2011; Felton and Tumlinson, 2008; Howe and Jander, 2008; Kessler and Baldwin, 2002; Maffei et al., 2012; Maffei et al., 2007; Mithöfer and Boland, 2012; Reymond, 2013; Wu and Baldwin, 2010). This relates to the recognition of attackers and the induction of signal transduction pathways, which is followed by transcriptomic changes and the induction of biosynthetic pathways leading to changes in plant phenotype. Most of this research has focused on interactions between a plant and one attacker, but over the past decade, studies of the interactive effects of the combined infestation of a plant by two attackers have been initiated (Dicke et al., 2009; Kessler and Baldwin, 2004; Rodriguez-Saona et al., 2010; Thaler et al., 2012; Voelckel and Baldwin, 2004; Zhang et al., 2013).



### Signal transduction pathways

Herbivorous insects produce oral secretions containing compounds that elicit plant responses (Bonaventure et al., 2011). The chemical nature of the active compounds is remarkably diverse and includes small organic compounds such as benzyl cyanide, fatty acid–amino acid conjugates, and proteins such as β-glucosidase (Maffei et al., 2012). The initial step in the elicitation process occurs with considerable specificity for the plant–insect combination studied. The recognition of herbivore elicitors by plant receptors initiates a cascade of responses, including changes in plasma membrane potential and activation of networks of kinases and phytohormones (Maffei et al., 2007). More recently, it has become apparent that insects may also produce so-called effectors that function to suppress the elicitor-triggered plant defense response, such as glucose oxidase in the interaction between *Helicoverpa zea* caterpillars and tobacco (Felton and Tumlinson, 2008; Maffei et al., 2012). Studies elucidating the regulatory mechanisms underpinning plant defense responses to insect herbivore attack have identified the central role of phytohormones. Three major plant hormones—jasmonic acid (JA), salicylic acid (SA), and ethylene (ET) (Fig 3A)—function in a complex regulatory network that is essential in herbivore-induced defense responses. Other hormones, such as cytokinins, abscisic acid, gibberellins, and auxin, likely also play a role in herbivore-induced defense signaling (Erb et al., 2012; Pieterse et al., 2012).

It is well documented that chewing herbivores and sap feeders induce different plant signaling pathways involving the three major phytohormones, JA, SA, and ET (Pieterse et al., 2012). Much less is known about signaling pathways involved in resistance against insects of other feeding guilds, such as leaf miners, stem borers, leaf folders, and gall-inducing herbivores. SA and ET signaling pathways are involved in the resistance of rice

plants to the leaf folder *Cnaphalocrocis medinalis* (Wang et al., 2011). Some leaf miners and gall-inducing insects modulate plant cytokinin levels, probably to manipulate the source–sink status of the infected tissues (reviewed in Erb et al. (2012) and Giron et al. (2013)). Feeding by gall-inducing insects increases auxin level but does not change JA level (Erb et al., 2012; Tooker and De Moraes, 2008). Insect eggs have been reported to induce plant responses via the SA signaling pathway (Reymond, 2013).

### *The salicylic acid pathway*

SA regulates induced plant responses against phloem-feeding insects and biotrophic pathogens (Glazebrook, 2005; Pieterse et al., 2012). In response to phloem-sucking insects, SA can be synthesized from chorismate through the isochorismate pathway (Wildemuth et al., 2001) and the phenylalanine ammonium lyase pathway (Dempsey et al., 2011). Accumulation of SA leads to the translocation of the positive regulatory protein nonexpressor of pathogenesis-related genes 1 (NPR1) to the nucleus. Regulation of the expression of SA-responsive genes occurs downstream of NPR1, which interacts with TGA-type transcription factors and additionally targets *WRKY* transcription factor genes (Wang et al., 2006). This results in the activation of defense gene expression and the production of pathogenesis-related (PR) proteins (Durrant and Dong, 2004).

### *The jasmonic acid/ethylene pathway*

JA is an important regulator of defense responses against chewing insects, necrotrophic pathogens, and cell content feeders such as spider mites and thrips (De Vos et al., 2005; Glazebrook, 2005; Kant et al., 2008; Pieterse et al., 2012). Upon herbivory, JA is produced via the octadecanoid pathway. In *Arabidopsis*, the enzyme jasmonoyl isoleucine conjugate synthase 1 (JAR1) activates JA by conjugating it to the amino acid isoleucine (Ile) to form JA-Ile (Staswick and Tiryaki, 2004). Binding of JA-Ile to the F-box protein coronatine-insensitive 1 (COI1) mediates the degradation of jasmonate ZIM domain (JAZ) repressor proteins (Thines et al., 2007). These proteins repress JA signaling by binding transcriptional activators such as MYC2. When the repression of JAZ proteins is lifted, JA-responsive genes are activated, including genes encoding JAZ proteins, resulting in a negative-feedback loop (Memelink, 2009). Two branches have been identified within the JA signaling pathway that act antagonistically (Pieterse et al., 2009; Pieterse et al., 2012). The MYC2 branch positively regulates the expression of wound-inducible JA-responsive marker genes such as *VEGETATIVE STORAGE PROTEIN 2* (*VSP2*) and *LIPOXYGENASE 2* (*LOX2*). In the ethylene response factor (ERF) branch of the JA pathway, JA and ET synergistically induce the expression of JA/ET-responsive transcription factors, including ERF1 and octadecanoid-responsive *Arabidopsis* 59 (ORA59), which positively regulate JA/ET-responsive genes such as *plant defensin 1.2* (*PDF1.2*) (Dombrecht et al., 2007; Lorenzo et al., 2004). The ERF branch is especially involved in induced defense against necrotrophic pathogens, whereas the MYC2 branch mediates defense against herbivorous insects (Pieterse et al., 2012).

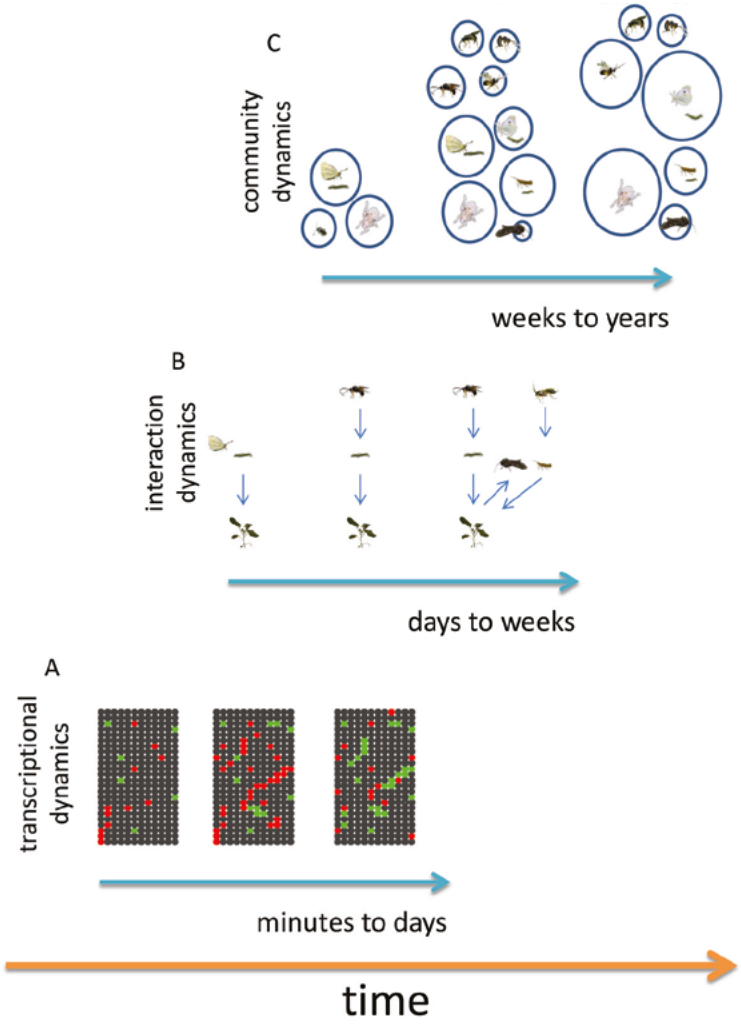


**Figure 3.** Schematic representation of dynamics at different levels of biological integration, each with its own timescale. Phytohormonal and transcriptional responses to herbivory at a scale of minutes to days (a). The tissue concentrations of the phytohormones jasmonic acid (JA), salicylic acid (SA), and ethylene (ET), which are involved in defense responses, change dynamically and exhibit crosstalk (arrows); their molecular structures are shown in red when increasing, in green when decreasing, and in black when constant. The dots represent genes in a heat map of gene transcription and are colored red when increasing, green when decreasing, and black when constant. Interactions among individual insects at different trophic levels at a scale of days to weeks (b). Community dynamics at a scale of weeks to years (c).

### *Phytohormonal crosstalk and its molecular mechanisms*

When a plant faces multiple herbivore attack, crosstalk may occur between the induced signaling pathways, with consequences for induced defense responses. Crosstalk between signaling pathways allows the plant to fine-tune its defense response to the specific attacker (Pieterse et al., 2012). For instance, induced defense is regulated through interconnection of

the JA, SA, and ET signal transduction pathways (Pieterse et al., 2012). Crosstalk between JA and SA signaling is mutually antagonistic, resulting in the prioritization of SA-dependent defense responses over JA-dependent responses or vice versa (Pieterse et al., 2012, Thaler et al., 2012). Molecular players that modulate this JA-SA crosstalk include mitogen-activated protein kinases (MAPKs), WRKY transcription factors, the regulatory protein NPR1, and other phytohormones (Pieterse et al., 2012). NPR1 is a major regulator of JA-SA crosstalk in *Arabidopsis*, and its effect is mediated by ET, which may have been induced by both biotic and abiotic stresses (Leon-Reyes et al., 2009). In contrast to JA-SA crosstalk, JA- and ET-dependent signaling pathways act synergistically in inducing plant defense responses (Pieterse et al., 2009).



Crosstalk between phytohormonal signaling pathways also allows herbivores to manipulate plant defenses for their own benefit (Pieterse and Dicke, 2007). Feeding by *Manduca sexta* caterpillars induced an ET burst and suppressed nicotine accumulation in tobacco plants (Kahl et al., 2000). It has been hypothesized that by activating the SA signaling pathway, phloem feeders suppress JA-dependent defenses to which phloem feeders are more sensitive (Moran et al., 2002; Zarate et al., 2007). Several recent studies have supported the interference of SA with JA-inducible defenses against chewing insects (Soler et al., 2012; Thaler et al., 2012; Zhang et al., 2013; Zhang et al., 2009), although phloem-feeding insects do not in all cases interfere with the defenses induced by chewing herbivores (Erb et al., 2010), which may be due to density effects or to differences between species.

### Transcriptomic changes in response to individual attackers and multiple attacks

Phytohormonal responses to herbivory result in transcriptional responses that have a high degree of specificity. Transcriptional responses depend on the feeding guild of the attacker and the phytohormonal signal signature that the attacker induces. For instance, attack by single insect species belonging to different feeding guilds resulted in the activation of specific sets of defense-related genes in *Arabidopsis* (De Vos et al., 2005). Different species of leaf-chewing herbivores that all induced JA in the plant still induced different transcriptomic changes (Bidart-Bouzat and Kliebenstein, 2011). These induced transcriptomic changes also differed from those induced by JA, most likely because each attacker activates more than one phytohormonal pathway. De Vos et al. (2005) hypothesized that the phytohormonal signal signature regulates the specific transcriptomic changes. Aphid feeding affected the expression of a substantially larger number of genes compared with feeding by caterpillars and thrips, and it tends to induce gene sets more similar to those induced by fungal or bacterial pathogens (De Vos et al., 2005). In *Nicotiana attenuata*, aphids suppressed more genes than chewing herbivores did, and aphids upregulated the expression of SA-dependent genes and suppressed the expression of JA-mediated genes (Heidel and Baldwin, 2004). Similar findings were recorded for the effects of feeding by caterpillars and aphids on tomato (*Solanum lycopersicum*) (Rodriguez-Saona et al., 2010).

***Transcriptomic changes in response to phloem-feeding insects***

Phloem-feeding insects, such as aphids and whiteflies, cause little damage to the plant tissue because they move their stylets in between plant cells on their way to the phloem, briefly puncturing but not killing cells along the way. SA accumulates in plants upon interactions with aphids and whiteflies, whereas activation of JA leads to resistance to phloem-feeding herbivores. Early transcriptional responses of *Arabidopsis* to *Brevicoryne brassicae* aphids were observed after 6 h, at which point a group of WRKY transcription factors were highly expressed. Genes involved in SA-dependent defense had a peak expression after 24 h of infestation. After 12 h of aphid infestation, the number of inducible genes expressed and the intensity of JA-inducible responses had already decreased (Kusnierczyk et al., 2008).



***Transcriptomic changes in response to chewing insects***

Plants respond to feeding by chewing insects very differently than they do to feeding by phloem-feeding insects (Bidart-Bouzat and Kliebenstein, 2011). Plant defense responses to chewing insects are regulated mainly by the JA signaling pathway, with ET playing an additional role (De Vos et al., 2005; Ehlting et al., 2008; Heidel and Baldwin, 2004; Reymond et al., 2004). The expression of hundreds of genes changes in response to caterpillar feeding (Ehlting et al., 2008; Reymond et al., 2004; Rodriguez-Saona et al., 2010; Voelckel and Baldwin, 2004; Zhang et al., 2013). Genes involved in signaling and secondary chemistry are commonly upregulated, whereas genes involved in photosynthesis and primary metabolism are often downregulated (Voelckel and Baldwin, 2004). The transcriptional patterns in response to caterpillar feeding are dynamic over time. For instance, a microarray analysis of *Arabidopsis* in response to feeding of *Plutella xylostella* larvae recorded strong upregulation of wound-response genes involved in octadecanoid biosynthesis over a 24-h period (Ehlting et al., 2008). However, SA also seems to be involved in the plant's response to *P. xylostella* feeding, as indicated by upregulation of *PR* genes after 24 h of feeding. Interestingly, *PR* genes are downregulated during early stages of *P. xylostella* feeding (Ehlting et al., 2008). Similar responses have been reported in other plant species as well. For example, in tomato, the transcription of *PR* genes was induced by caterpillar feeding (Kawazu et al., 2012; Rodriguez-Saona et al., 2010). In *N. attenuata*, feeding by various insect herbivores, including the chewing herbivores *Spodoptera exigua, Spodoptera littoralis, Trichoplusia ni and Manduca sexta* larvae resulted in increased SA levels (Diezel et al., 2009; Heidel and Baldwin, 2004). The increased SA levels were consistently correlated with the downregulation of photosynthetic genes (Heidel and Baldwin, 2004).

***Transcriptomic response patterns in response to multiple attacks***

The transcriptomic response to two attackers is far from an additive response to the two attackers individually (Voelckel and Baldwin, 2004). For instance, in tomato plants infested by aphids (*Macrosiphum euphorbiae*) and caterpillars (*S. exigua*), the aphids suppressed

27% of the genes regulated by caterpillars, whereas the caterpillars suppressed 66% of the genes regulated by aphids (Rodriguez-Saona et al., 2010). In *Arabidopsis*, infestation with the whitefly *Bemisia tabaci* suppresses the upregulation of a large number of genes induced by *P. xylostella* caterpillars (Zhang et al., 2013). The interactive effects of two attackers can uncover novel mechanisms. For instance, infestation of *Arabidopsis* plants by *P. rapae* caterpillars induced JA and ET; ET primed the plant for enhanced SA-dependent gene expression in response to infection by turnip crinkle virus (De Vos et al., 2006). Transcriptional interference is usually asymmetric. For instance, in *N. attenuata*, transcriptional changes induced by the mirid bug *Tupiocoris notatus* are more resistant to erasure by *M. sexta* caterpillars than vice versa (Voelckel and Baldwin, 2004). Transcriptomic changes occur in distinct patterns and involve large numbers of genes. Analyzing these patterns is usually done with multivariate statistics, but identifying how these transcriptomic changes affect the plant phenotype, especially which genes are responsible for the phenotypic effects and subsequent interactions with members of the insect community, requires a directed approach. In lima bean plants, feeding by *B. tabaci* whiteflies suppressed the induction of the plant's *ocimene synthase* gene, which encodes an enzyme mediating a rate-limiting step in the biosynthesis of the plant volatile (*E*)-β-ocimene in response to spider-mite feeding. (*E*)-β-Ocimene mediates the attraction of a predatory mite that preys on the spider mite, and whitefly feeding resulted in a reduced attraction of the predatory mite to volatiles from spider-mite-infested plants (Zhang et al., 2009).



## How to link subcellular mechanisms underlying inducible plant phenotypes to community dynamics

Changes in plant phenotype and their consequences for the plant's interactions with members of the associated insect community take place at very different timescales. Community development takes place on a timescale of weeks to (for perennial woody plants) years, and is based on interactions between individuals that take place on a timescale of days to weeks. These interactions between individuals are affected by changes in the plant phenotype (timescale of hours to days) that are based on transcriptomic changes at a timescale of minutes to days (Fig 3). The different rates at which changes develop at different levels of biological complexity complicate linking these changes causally. For instance, the transcriptome of *N. attenuata* responds specifically to different herbivore infestations within 24 h, but this difference disappears after 5 days (Voelckel and Baldwin, 2004). Linking the transcriptomic response within the first 24 h to community responses at a timescale of weeks to years requires detailed knowledge of how individual species in the community respond to the plastic plant phenotype.

Although understanding how complex molecular changes modulate responses at the community level is a major challenge, detailed knowledge of subcellular mechanisms

can provide tools to address this challenge. For instance, knowledge on the involvement of phytohormones can be used to mimic herbivory through the application of a phytohormone. Because JA is one of the major phytohormones involved in plant responses to insect herbivory, it is an interesting initial candidate to manipulate. Pharmacological application of JA to tomato plants has season-long effects on community composition in terms of herbivorous and carnivorous insects. For instance, the abundance of herbivores was reduced and herbivore size was smaller, and these effects on herbivores subsequently affected the performance of predators and parasitoids (Thaler, 1999; Thaler, 2002; Thaler et al., 2001). Applying a single phytohormone at one time point is still a crude method, however, because herbivory results in a dynamic phytohormonal response (Pieterse et al., 2012). Pharmacological applications may be made with different phytohormones at different time points (Koornneef et al., 2008), but we are not aware of any studies that have investigated the effects of such combinations of applications on community development.



A more accurate approach is to use genetic tools, e.g., by using plants that have been silenced in a single gene involved in the plant's induced response. *N. attenuata* plants in which a gene encoding for the enzyme lipoxygenase, which mediates the first rate-limiting step in JA biosynthesis, had been silenced were more susceptible to adapted herbivores and attracted novel herbivore species that normally do not feed or reproduce on this plant (Kessler et al., 2004). Silencing a gene is quite a drastic manipulation. In nature, plant genotypes more likely differ in relative expression of particular genes, so it will be interesting to monitor community development on different genotypes whose genomes have been (partially) genetically characterized. Experiments with genotypes that have not been genetically characterized showed that plant genotypes that differ in secondary metabolites result in considerable variation in community dynamics (Newton et al., 2009b; Poelman et al., 2009). Community development on different genotypes may converge when the genotypes have been exposed to an early-season specialist herbivore (Poelman et al., 2008; Poelman et al., 2010). Because plants in nature are rarely free of herbivory, community dynamics on plants subjected to herbivory are highly relevant to understanding how plant phenotype affects community dynamics.

Data on community development may be linked to transcriptional responses of plants under field conditions (Broekgaarden et al., 2010), but this is still far from providing information on the causal links because of the different timescales. Transcriptional responses on a timescale of minutes to days result in a cascade of responses that lead to a dynamic change in plant phenotype. Studying the links between transcriptional dynamics, phenotypic dynamics, and community dynamics requires taking a systems approach that includes experiments in combination with modeling to connect the networks at different levels of biological integration, i.e., the transcriptomic network, the metabolomic network, and the species interaction network (Keurentjes et al., 2011).

## Sequential changes in herbivore-induced phenotype and community dynamics

Plant–insect interactions represent intricate networks at all levels of biological complexity. These networks consist of hundreds of interacting species at the community level, tens to hundreds of individual insects interacting with a single plant individual, hundreds of plant chemicals that are the product of biosynthetic networks, and hundreds of genes that are regulated by an interacting network of phytohormones. Each of these networks has its own dynamics, and the transcriptomic network that results from herbivore attack affects the biosynthetic network that underlies the change in plant phenotype, which affects interactions with members of the community and consequently community dynamics. Although a systems approach to linking these complex networks at different levels of biological integration will be a major challenge (Keurentjes et al., 2011), interesting building blocks are available at the community level with some initial links to knowledge at the mechanistic level. Community dynamics result from sequential processes in which the first herbivore's modification of the plant's phenotype then has consequences for the interactions of the plant with subsequent herbivores (Erb et al., 2011; Poelman et al., 2008; Poelman et al., 2010; Viswanathan et al., 2007). The interaction of a second herbivore with the new plant phenotype may modulate processes at the (sub)cellular level in terms of phytohormonal and transcriptional patterns (Poelman et al., 2008; Rodriguez-Saona et al., 2010; Voelckel and Baldwin, 2004; Zhang et al., 2013), further affecting the plant's phenotype and its interactions with subsequent community members (Dicke et al., 2009; Van Zandt and Agrawal, 2004; Zhang et al., 2013; Zhang et al., 2009). The arrival of these new community members, which now also start to interact with the plant, sets a new round in motion, and so on.

This set of interactions—an herbivore inducing a phenotypic change that then affects subsequent herbivores on the same plant, mediated by induced plant traits—has been termed a trait-mediated interaction unit (TMIU). A TMIU consists of an inducing insect and a plant that mediates the interaction with a second, responding herbivore (Utsumi et al., 2010). TMIUs are linked sequentially. This is the case when, for example, a responding herbivore itself becomes an inducer (Utsumi et al., 2010), which may happen on both spatial and temporal scales. A spatial chain reaction occurs when the responding herbivore changes its behavior and moves to another plant or plant part (Bukovinszky et al., 2010; Utsumi et al., 2010) or when responses to feeding herbivores affect herbivores elsewhere on the plant through systemic responses (Erb et al., 2011, Utsumi et al., 2010). A temporal chain reaction occurs when the responding herbivore later returns to the same plant as an inducer (Underwood, 2012) or when the altered plant phenotype affects the performance or population density of the responder, thereby affecting the plant it feeds on (Utsumi et al., 2010, Van Zandt and Agrawal, 2004).

In fact, several TMIUs might be linked throughout the season, creating a complex indirect interaction web. The resulting cascade shapes the insect community associated with a plant, depending on the first inducing herbivores that arrive on the plant (Poelman et al., 2008; Van Zandt and Agrawal, 2004; Viswanathan et al., 2007). For instance, on milkweed plants, the identity of the first herbivore early in the season has considerable effects on community development throughout the season (Van Zandt and Agrawal, 2004). On *B. oleracea* plants, an early-season, one-week-long infestation by two *P. rapae* caterpillars affected community dynamics throughout the growing season of the plants, with the community on the treated plants comprising more specialist insects than the community on the control plants did (Poelman et al., 2008; Poelman et al., 2010). Such cascades may be caused by direct effects of an inducing herbivore on the suitability of the plant to other herbivores and indirect effects of initiating herbivores on the interaction between two or more subsequent herbivores. Herbivores in a TMIU do not all influence the subsequent interactions in the same way, and this may depend on herbivore traits such as feeding guild (Bidart-Bouzat and Kliebenstein, 2011; Howe and Jander, 2008). Three mechanisms have been proposed by which a plant's physiological response is directed to (a subset of) certain herbivores: priority effects, overriding effects, and canalization, all of which can be linked to phenomena uncovered at the subcellular level.



### Priority effects, overriding effects, and canalization

Priority effects occur when a plant response depends on the order of herbivore arrival on a plant (Miller-Pierce and Preisser, 2012)—for example, when the interaction between two herbivores is asymmetrical (Erb et al., 2011; Miller-Pierce and Preisser, 2012; Poelman et al., 2008; Soler et al., 2012). Asymmetry in these interactions is predominant (Kaplan and Denno, 2007), and priority effects are therefore expected to be important in shaping interaction cascades. These asymmetrical priority effects can have several underlying mechanisms, such as competition between the herbivores (Kaplan and Denno, 2007; Miller-Pierce and Preisser, 2012), which has different outcomes depending on which insect comes first. The kinetics of plant defenses may underlie this. For example, the production of induced plant defense compounds might depend on the sequence of herbivore arrival and can have a larger effect on either the first or the subsequently arriving herbivore (Erb et al., 2011; Viswanathan et al., 2005). Priority effects may also be mediated by crosstalk between different plant defense pathways, such as the JA-SA crosstalk (Pieterse et al., 2012; Thaler et al., 2012).

Overriding effects occur when the inducing effects of one herbivore are overruled by another herbivore on the same plant (Erb et al., 2011; Van Zandt and Agrawal, 2004). For example, the effects of initial damage to a milkweed plant by monarch caterpillars (*Danaus plexippus*) disappeared when the plant was colonized by other herbivores later

in the season (Van Zandt and Agrawal, 2004). Moreover, the plant response can also be redirected (Soler et al., 2012; Voelckel and Baldwin, 2004) or enhanced (Poelman et al., 2008) following the arrival of subsequent herbivores. Underlying mechanisms may involve irreversible phenotypic changes, such as morphological changes or overriding effects of one signaling pathway on another (Pieterse et al., 2012).

Canalization occurs when a first herbivore alone determines the plant's response, regardless of subsequently arriving herbivores (Thaler et al., 2002; Utsumi et al. 2010; Viswanathan et al., 2007; Viswanathan et al., 2005). This effect reduces the plant's ability to be flexible in its response to the herbivore community present at any given point in time, and consequently may affect the development of the herbivore community composition throughout the season. For example, flea beetles affected the number of conspecifics or tortoise beetles throughout the season when arriving first on a plant, irrespective of whether they were followed by tortoise beetles (Viswanathan et al., 2007). Underlying mechanisms may include strong and irreversible effects of the phytohormonal signaling in response to the first herbivore or the rapid induction of biosynthetic pathways that result in persistent changes in the plant's phenotype.



### Trait-mediated interaction networks and carnivorous insects

The above discussion of trait-mediated interaction networks considered only herbivores in the ecological interactions. However, the third trophic level, consisting of predators and parasitoids of herbivores, also affects the interaction between inducing and responding herbivores (Utsumi et al., 2010; Van Veen et al., 2006). Combinations of multiple herbivores can induce the emission of different blends of plant volatiles (Dicke et al., 2009) and thus may attract different predators or parasitoids (Schoonhoven et al., 2005). These predators or parasitoids not only decrease the herbivore population by preying on the insects that initially induced the volatiles (Utsumi et al., 2010; Xiao et al., 2012; Zhang et al., 2009) but can also affect other insects, such as herbivores, pollinators, and hyperparasitoids (Dicke and Baldwin, 2010; Poelman et al., 2012). The events at different moments in time may also be linked, for example, when a predator that is attracted to a plant infested by a first herbivore also preys on other herbivores arriving simultaneously or subsequently on the plant. Different interaction units can occur on a spatial scale as well, when predators induce behavioral changes in herbivores, after which the herbivores move to other plants or plant parts (Utsumi et al., 2010).

Because herbivores that are affected by predators and parasitoids can influence the subsequent herbivore community in a cascading manner through priority effects, overruling effects, or canalization, the third trophic level greatly increases the complexity of interactions within a plant–insect community. This is particularly the case when considering multiple initiating herbivores (Zhang et al., 2013; Zhang et al., 2009).

In summary, interactions between insects associated with a plant are influenced by several factors. The type and sequence of multiple herbivores determine the plant's response, which consequently affects herbivores that subsequently colonize the plant. These secondary herbivores or attracted predators may become inducers in the next plant-mediated interaction unit, which causes a cascade of interactions throughout the insect community.

## Future perspectives



The fact that plants are phenotypically plastic in response to herbivore attack contributes to the complexity of plant–insect interactions. For instance, phenotypic plasticity underlies interspecific competition between herbivores at different temporal and spatial scales (Denno et al., 1995; Kaplan and Denno, 2007). It is important to realize that a plant's genotype determines not only constitutive plant traits but also inducible plant responses, such as the production of metabolites or structural changes. The extent to which constitutive or inducible traits affect plant–insect interactions affects the relative importance of the inducible and the constitutive phenotype for the influence on community dynamics (Poelman et al., 2008; Whitham et al., 2012).

In this review, we have focused on the consequences of direct and indirect effects of inducible plant traits on community processes, with a focus on herbivorous and carnivorous insects. Herbivorous insects are connected by both local and systemic plant-mediated interactions. Systemic effects may involve both roots and shoots (Soler et al., 2013) or leaves and flowers (Kessler et al., 2011; Lucas-Barbosa et al., 2011). We have focused on aboveground plant vegetative tissues because most information on community processes is available for insect communities associated with vegetative plant shoots. However, similar systemic effects are expected when including the belowground tissues (Soler et al., 2013). Including belowground interactions will be important, even when it further increases the complexity of the interactions and therefore the difficulty of understanding the effects of a phenotypically plastic plant on the development of the associated community. The situation is likely to differ between vegetative and flowering plants because of the major physiological changes that occur during the transition from the vegetative to the reproductive stage. A comparison of vegetative and flowering plants and their associated communities will be interesting to address the different selection forces that these different developmental stages are subjected to.

For the sake of simplicity, we have limited this review to plant–insect interactions. Although insects are the most speciose group within the macrobiome associated with plants (Schoonhoven et al., 2005), there is also a speciose microbiome associated with plants (Mendes et al., 2011) that represents species with many additional ecological

functions, such as pathogens, rhizobia, mycorrhizae, and nonpathogenic rhizobacteria. There is extensive information at the mechanistic, (sub)cellular level for plant–microbe interactions (Pieterse et al., 2012), but knowledge of the community processes of microbes associated with plants is much less developed. Nevertheless, it is becoming clear that pathogenic and symbiotic microbes can influence and structure insect communities on plants (Pineda et al., 2010; Tack and Dicke, 2013). Thus, involving the microbiome in future studies will significantly enhance our understanding of plant–insect interactions. The extensive information on subcellular processes for plant–microbe interactions provides an excellent starting point to manipulate plants via microbes to study the consequences for insect communities. However, including microbial community processes will provide an important new challenge related to the identification of microbes associated with plants (Mendes et al., 2011).



Investigating the effects of plants on community development is already a complex task, and unraveling the mechanisms that underlie the community dynamics throughout the season is a significant challenge as well. With a community that, in the case of long-lived plants, can consist of hundreds of species, the number of species combinations involved in plant-mediated interactions seems too large to handle. However, phenological data and natural history data for the system under study may provide a basis for choices that are relevant to the natural situation. For instance, early-season herbivores that predictably occur in the system may have a prominent effect on plant phenotype that is worth focusing on initially. Furthermore, analyzing community dynamics data through statistical modeling approaches may result in the identification of key species in the community (Keurentjes et al., 2011). Such species and the species they interact with may then be the focus of initial studies on underlying mechanisms. Herbivorous insects will be the first group of insects to focus on. When key herbivore species have been selected for such studies, relevant parasitoids and predators should be included next, because their presence and activities affect herbivore behavior (Thaler et al., 2012), population growth (Van Veen et al., 2005), and interactions with plants (Poelman et al., 2011). Again, natural history data may guide the selection of the first species to include in these studies.

The complexity of plant–insect community dynamics and the underlying mechanisms may be overwhelming, and it may seem impossible to understand the processes that shape these speciose and dynamic ecological systems. Rather than stepping back, this complexity should invite directed studies to investigate the ecological processes as well as their underlying mechanisms. Through these studies, we are likely to make small but significant steps toward unraveling how plants influence insect communities. When this has been completed for several different systems, ecological generalities may be identified, and mechanistic knowledge will then allow directed experimental studies to test these generalities. These studies will then enable important progress in understanding interactions between the insects and plants that are so dominant on this planet.

## Summary points

1. Plants are members of biodiverse communities consisting of tens to hundreds of species.
2. The insect community associated with plants consists of herbivores at the second trophic level and (hyper)parasitoids and predators at the third, fourth, and higher trophic levels.
3. A plant's phenotypic traits, as determined by its genotype, influence the interactions of the plant with members of the associated community and consequently the community dynamics.
4. A plant's phenotype is highly plastic: Herbivory induces changes in the plant's phenotype, which then influence the plant's interactions with members of the associated community and lead to plant-mediated interactions between community members, such as competition and facilitation.
5. Herbivory induces phytohormonal signaling and transcriptomic rearrangements (timescale of minutes to days) that lead to biosynthetic changes that affect the plant phenotype (timescale of hours to days), with consequences for the plant's interactions with community members (timescale of days to weeks).
6. Plant responses to herbivores exhibit a considerable degree of specificity. Moreover, the response to two attackers is far from an additive response to the two attackers individually; rather, it involves a strong interaction component that leads to suppression or enhancement of the responses to each herbivore alone, e.g., through phytohormonal crosstalk.
7. The first herbivore-induced change in plant phenotype affects the interactions with subsequently arriving herbivores, which then further affect the phenotype in an interactive way. This sequential process determines community dynamics on a timescale of weeks to years. Thus, the first herbivore that attacks a plant can significantly influence the community dynamics on that plant.
8. Linking herbivore-induced changes in plant phenotype to the ecological consequences that occur at very different timescales is an important multidisciplinary challenge that will provide a comprehensive understanding of how plants interact with their associated communities.

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## References

Agrawal AA (2000) Specificity of induced resistance in wild radish: causes and consequences for two specialist and two generalist caterpillars. Oikos 89: 493–500.

Agrawal AA (2005) Natural selection on common milkweed (*Asclepias syriaca*) by a community of specialized insect herbivores. Evolutionary Ecology Research 7: 651–667.

Agrawal AA, Karban R, Colfer RG (2000) How leaf domatia and induced plant resistance affect herbivores, natural enemies and plant performance. Oikos 89: 70–80.

Ali JG, Agrawal AA (2012) Specialist versus generalist insect herbivores and plant defense. Trends in Plant Science 17: 293–302.

Andow DA (1991) Vegetational diversity and arthropod population response. Annual Review of Entomology 36: 561–586.

Baldwin IT (2012) Training a new generation of biologists: the genome-enabled field biologists. Proceedings of the American Philosophical Society 156: 205–214.

Barbour RC, O'Reilly-Wapstra JM, De Little DW, Jordan GJ, Steane DA, Humphreys JR, Bailey JK, Whitham TG, Potts, BM (2009) A geographic mosaic of genetic variation within a foundation tree species and its community-level consequences. Ecology 90: 1762–1772.

Benrey B, Denno RF (1997) The slow growth high mortality hypothesis: a test using the cabbage butterfly. Ecology 78: 987–999.

Bidart-Bouzat MG, Kliebenstein D (2011) An ecological genomic approach challenging the paradigm of differential plant responses to specialist versus generalist insect herbivores. Oecologia 167: 677–689.

Bonaventure G, Van Doorn A, Baldwin IT (2011) Herbivore-associated elicitors: FAC signaling and metabolism. Trends in Plant Science 16: 294–299.

Braasch J, Wimp GM, Kaplan I (2012) Testing for phytochemical synergism: arthropod community responses to induced plant volatile blends across crops. Journal of Chemical Ecology 38: 1264–1275.

Broekgaarden C, Poelman EH, Voorrips RE, Dicke M, Vosman B (2010) Intraspecific variation in herbivore community composition and transcriptional profiles in field-grown *Brassica oleracea* cultivars. Journal of Experimental Botany 61: 807–819.

Bukovinszky T, Gols R, Kamp A, De Oliveira-Domingues F, Hamback PA, Jongema Y, Bezemer TM, Dicke M, Van Dam NM, Harvey JA (2010) Combined effects of patch size and plant nutritional quality on local densities of insect herbivores. Basic and Applied Ecology 11: 396–405.

Bukovinszky T, Gols R, Smid HM, Bukovinskine Kiss G, Dicke M, Harvey JA (2012) Consequences of constitutive and induced variation in the host's food plant quality for parasitoid larval development. Journal of Insect Physiology 58: 367–375.

Bukovinszky T, Poelman EH, Gols R, Prekatsakis G, Vet LEM, Harvey JA, Dicke M (2009) Consequences of constitutive and induced variation in plant nutritional quality for immune defence of a herbivore against parasitism. Oecologia 160: 299–308.

Bukovinszky T, Van Veen FJF, Jongema Y, Dicke M (2008) Direct and indirect effects of resource quality on food web structure. Science 319: 804–807.

Cerrudo I, Keller MM, Cargnel MD, Demkura PV, De Wit M, Patitucci MS, Pierik R, Pieterse CMJ, Ballaré CL (2012) Low red/far-red ratios reduce *Arabidopsis* resistance to *Botrytis cinerea* and jasmonate responses via a COI1-JAZ10-dependent, salicylic acid-independent mechanism. Plant Physiology 158: 2042–2052.

D'Alessandro M, Turlings TCJ (2006) Advances and challenges in the identification of volatiles that mediate interactions among plants and arthropods. Analyst 131: 24–32.

De Rijk M, Dicke M, Poelman EH (2013) Foraging behaviour by parasitoids in multiherbivore communities. Animal Behaviour 85: 1517–1528.

De Vos M, Van Oosten VR, Van Poecke RMP, Van Pelt JA, Pozo MJ, Mueller MJ, Buchala AJ, Métraux JP, Van Loon LC, Dicke M, Pieterse CMJ (2005) Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. Molecular Plant-Microbe Interactions 18: 923–937.

De Vos M, Van Zaanen W, Koornneef A, Korzelius JP, Dicke M, Van Loon LC, Pieterse CMJ (2006) Herbivore-induced resistance against microbial pathogens in *Arabidopsis*. Plant Physiology 142: 352–363.

Dempsey DA, Vlot AC, Wildermuth MC, Klessig DF (2011) Salicylic acid biosynthesis and metabolism. Arabidopsis Book 9: e0156.

Denno RF, McClure MS, Ott JR (1995) Interspecific interactions in phytophagous insects: competition reexamined and resurrected. Annual Review of Entomology 40: 297–331.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the "cry for help." Trends in Plant Science 15: 167–175.

Dicke M, Van Loon JJA, Soler R (2009) Chemical complexity of volatiles from plants induced by multiple attack. Nature Chemical Biology 5: 317–324.

Diezel C, von Dahl CC, Gaquerel E, Baldwin IT (2009) Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. Plant Physiology 150: 1576–1586.

Dombrecht B, Xue GP, Sprague SJ, Kirkegaard Ja, Ross JJ, Reid JB, Fitt GP, Sewelam N, Schenk PM, Manners JM, Kazan K (2007) MYC2 differentially modulates diverse jasmonate-dependent functions in *Arabidopsis*. The Plant Cell 19: 2225–2245.

Durrant WE, Dong X (2004) Systemic acquired resistance. Annual Review of Phytopathology 42: 185–209.

Dussourd DE (1995) Entrapment of aphids and whiteflies in lettuce latex. Annals of the Entomological Society of America 88: 163–172.

Ehlting J, Chowrira SG, Mattheus N, Aeschliman DS, Arimura G, Bohlmann J (2008) Comparative transcriptome analysis of *Arabidopsis thaliana* infested by diamond back moth (*Plutella xylostella*) larvae reveals signatures of stress response, secondary metabolism, and signalling. BMC Genomics 9: 154.

Erb M, Foresti N, Turlings TCJ (2010) A tritrophic signal that attracts parasitoids to host-damaged plants withstands disruption by non-host herbivores. BMC Plant Biology 10: 247.

Erb M, Meldau S, Howe GA (2012) Role of phytohormones in insect-specific plant reactions. Trends in Plant Science 17: 250–259.

Erb M, Robert CAM, Hibbard BE, Turlings TCJ (2011) Sequence of arrival determines plant-mediated interactions between herbivores. Journal of Ecology 99: 7–15.

Felton GW, Tumlinson JH (2008) Plant-insect dialogs: complex interactions at the plant-insect interface. Current Opinion in Plant Biology 11: 457–463.

Fritz RS, McDonough SE, Rhoads AG (1997) Effects of plant hybridization on herbivore-parasitoid interactions. Oecologia 110: 360–367.

Giron D, Frago E, Glevarec G, Pieterse CMJ, Dicke M (2013) Cytokinins as key regulators in plant-microbe-insect interactions: connecting plant growth and defence. Functional Ecology 27: 599–609.

Glazebrook J (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annual Review of Phytopathology 43: 205–227.

Gols R, Bukovinszky T, Van Dam NM, Dicke M, Bullock JM, Harvey JA (2008a) Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild *Brassica* populations. Journal of Chemical Ecology 34: 132–143.

Gols R, Harvey JA (2009) Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. Phytochemistry Reviews 8: 187–206.

Gols R, Wagenaar R, Bukovinszky T, Van Dam NM, Dicke M, Bullock JM, Harvey JA (2008b) Genetic variation in defense chemistry in wild cabbages affects herbivores and their endoparasitoids. Ecology 89: 1616–1626.

Hartley SE, Gange AC (2009) Impacts of plant symbiotic fungi on insect herbivores: mutualism in a multitrophic context. Annual Review of Entomology 54: 323–342.

Harvey JA, Van Dam NM, Raaijmakers CE, Bullock JM, Gols R (2011) Tri-trophic effects of inter- and intra-population variation in defence chemistry of wild cabbage (*Brassica oleracea*). Oecologia 166: 421–431.

Harvey JA, Wagenaar R, Bezemer TM (2009) Interactions to the fifth trophic level: secondary and tertiary parasitoid wasps show extraordinary efficiency in utilizing host resources. Journal of Animal Ecology 78: 686–692.

Haukioja E (1980) On the role of plant defences in the fluctuation of herbivore populations. Oikos 35: 202–213.

Heidel AJ, Baldwin IT (2004) Microarray analysis of salicylic acid- and jasmonic acid-signalling in responses of *Nicotiana attenuata* to attack by insects from multiple feeding guilds. Plant, Cell and Environment 27: 1362–1373.

Heil M (2008) Indirect defence via tritrophic interactions. New Phytologist 178: 41–61.

Heil M, Koch T, Hilpert A, Fiala B, Boland W, Linsenmair KE (2001) Extrafloral nectar production of the ant-associated plant, *Macaranga tanarius*, is an induced, indirect, defensive response elicited by jasmonic acid. Proceedings of the American Philosophical Society USA 98: 1083–1088.

Hilker M, Meiners T (2010) How do plants "notice" attack by herbivorous arthropods? Biological Reviews 85: 267–280.

Hopkins RJ, Van Dam NM, Van Loon JJA (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annual Review of Entomology 54: 57–83.

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annual Review of Plant Biology 59: 41–66.

Hunter MD, Price PW (1992) Playing chutes and ladders: heterogeneity and the relative roles of bottom-up and top-down forces in natural communities. Ecology 73: 724–732.

Johnson MTJ (2008) Bottom-up effects of plant genotype on aphids, ants, and predators. Ecology 89: 145–154.

Johnson MTJ, Agrawal AA (2005) Plant genotype and environment interact to shape a diverse arthropod community on evening primrose (*Oenothera biennis*). Ecology 86: 874–885

Johnson MTJ, Agrawal AA (2007) Covariation and composition of arthropod species across plant genotypes of evening primrose (*Oenothera biennis*). Oikos 116: 941–956.

Johnson MTJ, Lajeunesse MJ, Agrawal AA (2006) Additive and interactive effects of plant genotypic diversity on arthropod communities and plant fitness. Ecology Letters 9: 24–34.

Kahl J, Siemens DH, Aerts RJ, Gabler R, Kuhnemann F, Preston CA, Baldwin IT (2000) Herbivore-induced ethylene suppresses a direct defense but not a putative indirect defense against an adapted herbivore. Planta 210: 336–342.

Kant MR, Sabelis MW, Haring MA, Schuurink RC (2008) Intraspecific variation in a generalist herbivore accounts for differential induction and impact of host plant defences. Proceedings of the Royal Society B: Biological Sciences 275: 443–452.

Kaplan I, Denno RF (2007) Interspecific interactions in phytophagous insects revisited: a quantitative assessment of competition theory. Ecology Letters 10: 977–994.

Karban R, Agrawal AA (2002) Herbivore offense. Annual Review of Ecology, Evolution and Systematics 33: 641–664.

Karban R, Agrawal AA, Mangel M (1997) The benefits of induced defenses against herbivores. Ecology 78: 1351–1355.

Karban R, Baldwin IT (1997) Induced responses to herbivory. Chicago: Chicago Univ. Press.

Kareiva P, Sahakian R (1990) Tritrophic effects of a simple architectural mutation in pea plants. Nature 345: 433–434.

Kawazu K, Mochizuki A, Sato Y, Sugeno W, Murata M, Seo S, Mitsuhara I (2012) Different expression profiles of jasmonic acid and salicylic acid inducible genes in the tomato plant against herbivores with various feeding modes. Arthropod-Plant Interactions 6 (2): 221–230.

Kazana E, Pope TW, Tibbles L, Bridges M, Pickett JA, Bones AM, Powell G, Rossiter JT (2007). The cabbage aphid: a walking mustard oil bomb. Proceedings of the Royal Society B: Biological Sciences 274: 2271–2277.

Keith AR, Bailey JK, Whitham TG (2010) A genetic basis to community repeatability and stability. Ecology 91: 3398–3406.

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annual Review of Plant Biology 53: 299–328.

Kessler A, Baldwin IT (2004) Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco *Nicotiana attenuata*. The Plant Journal 38: 639–649.

Kessler A, Halitschke R, Baldwin IT (2004) Silencing the jasmonate cascade: induced plant defenses and insect populations. Science 305: 665–668.

Kessler A, Halitschke R, Poveda K (2011) Herbivory-mediated pollinator limitation: negative impacts of induced volatiles on plant-pollinator interactions. Ecology 92: 1769–1780.

Keurentjes JJB, Angenent GC, Dicke M, Dos Santos V, Molenaar J, Van der Putten WH, De Ruiter PC, Struik PC, Thomma B (2011) Redefining plant systems biology: from cell to ecosystem. Trends in Plant Science 16: 183–190.

Koornneef A, Leon-Reyes A, Ritsema T, Verhage A, Den Otter FC, Van Loon LC, Pieterse CMJ (2008) Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. Plant Physiology 147: 1358–1368.

Kos M, Broekgaarden C, Kabouw P, Lenferink KO, Poelman EH, Vet LEM, Dicke M, Van Loon JJA (2011) Relative importance of plant-mediated bottom-up and top-down forces on herbivore abundance on *Brassica oleracea*. Functional Ecology 25: 1113–1124.

Kusnierczyk A, Winge P, Jørstad TS, Troczyńska J, Rossiter JT, Bones AM (2008) Towards global understanding of plant defence against aphids–timing and dynamics of early *Arabidopsis* defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. Plant, Cell and Environment 31: 1097–1115.

Leimu R, Koricheva J (2006) A meta-analysis of genetic correlations between plant resistances to multiple enemies. The American Naturalist 168: E15–37.

Leon-Reyes A, Spoel SH, De Lange ES, Abe H, Kobayashi M, Tsuda S, Millenaar FF, Welschen RAM, Ritsema T, Pieterse CMJ (2009) Ethylene modulates the role of *NONEXPRESSOR OF PATHOGENESIS-RELATED GENES1* in cross talk between salicylate and jasmonate signaling. Plant Physiology 149: 1797–1809.

Lorenzo O, Chico JM, Sa JJ (2004) *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. The Plant Cell 16: 1938–1950.

Lucas-Barbosa D, Van Loon JJA, Dicke M (2011) The effects of herbivore-induced plant volatiles on interactions between plants and flower-visiting insects. Phytochemistry 72: 1647–1654.

Maffei ME, Arimura G, Mithöfer A (2012) Natural elicitors, effectors and modulators of plant responses. Natural Product Reports 29: 1288–1303.

Maffei ME, Mithöfer A, Boland W (2007) Before gene expression: early events in plant-insect interaction. Trends in Plant Science 12: 310–316.

McCormick AC, Unsicker SB, Gershenzon J (2012) The specificity of herbivore-induced plant volatiles in attracting herbivore enemies. Trends in Plant Science 17: 303–310.

Memelink J (2009) Regulation of gene expression by jasmonate hormones. Phytochemistry 70: 1560–1570.

Mendes R, Kruijt M, De Bruijn I, Dekkers E, Van der Voort M, Schneider JH, Piceno YM, DeSantis TZ, Andersen GL, Bakker PA, Raaijmakers JM (2011) Deciphering the rhizosphere microbiome for disease-suppressive bacteria. Science 332: 1097–1100.

Miller-Pierce MR, Preisser EL (2012) Asymmetric priority effects influence the success of invasive forest insects. Ecological Entomology 37: 350–358.

Mithöfer A, Boland W (2012) Plant defense against herbivores: chemical aspects. Annual Reviews of Plant Biology 63: 431–450.

Moran PJ, Cheng Y, Cassell JL, Thompson GA (2002) Gene expression profiling of *Arabidopsis thaliana* in compatible plant-aphid interactions. Archives of Insect Biochemistry and Physiology 51: 182–203.

Müller C (2009) Interactions between glucosinolate-and myrosinase-containing plants and the sawfly *Athalia rosae*. Phytochemistry Reviews 8: 121–134.

Newton EL, Bullock JM, Hodgson DJ (2009a) Bottom-up effects of glucosinolate variation on aphid colony dynamics in wild cabbage populations. Ecological Entomology 34: 614–623.

Newton EL, Bullock JM, Hodgson DJ (2009b) Glucosinolate polymorphism in wild cabbage (*Brassica oleracea*) influences the structure of herbivore communities. Oecologia 160: 63–76.

Ode PJ (2006) Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. Annual Review of Entomology 51: 163–185.

Ohgushi T (2005) Indirect interaction webs: herbivore-induced effects through trait change in plants. Annual Review of Ecology, Evolution and Systematics 36: 81–105.

Ohgushi T (2008) Herbivore-induced indirect interaction webs on terrestrial plants: the importance of non-trophic, indirect, and facilitative interactions. Entomologia Experimentalis et Applicata 128: 217–229.

Pieterse CMJ, Dicke M (2007) Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends in Plant Science 12: 564–569.

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nature Chemical Biology 5: 308–316.

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal modulation of plant immunity. Annual Review of Cell and Developmental Biology 28: 489–521.

Pineda A, Zheng SJ, Van Loon JJA, Pieterse CMJ, Dicke M (2010) Helping plants to deal with insects: the role of beneficial soil-borne microbes. Trends in Plant Science 15: 507–514.

Poelman EH, Broekgaarden C, Van Loon JJA, 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 EH, Bruinsma M, Zhu F, Weldegergis BT, Boursault AE, Jongema Y, Van Loon JJA, Vet LEM, Harvey JA, Dicke M (2012) Hyperparasitoids use herbivore-induced plant volatiles to locate their parasitoid host. PLoS Biology 10: e1001435.

Poelman EH, Van Dam NM, Van Loon JJA, Vet LEM, Dicke M (2009) Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores. Ecology 90: 1863–1877.

Poelman EH, Van Loon JJA, Dicke M (2008) Consequences of variation in plant defense for biodiversity at higher trophic levels. Trends in Plant Science 13: 534–541.

Poelman EH, Van Loon JJA, Van Dam NM, Vet LEM, Dicke M (2010) Herbivore-induced plant responses in *Brassica oleracea* prevail over effects of constitutive resistance and result in enhanced herbivore attack. Ecological Entomology 35: 240–247.

Poelman EH, Zheng SJ, Zhang Z, Heemskerk NM, Cortesero AM, Dicke M (2011) Parasitoid-specific induction of plant responses to parasitized herbivores affects colonization by subsequent herbivores. Proceedings of the National Academy of Sciences USA 108: 19647–19652.

Ponzio C, Gols R, Pieterse CMJ, Dicke M (2013) Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens. Functional Ecology 27: 587–598.

Price PW, Bouton CE, Gross P, McPheron BA, Thompson JN, Weis AE (1980) Interactions among three trophic levels: influence of plant on interactions between insect herbivores and natural enemies. Annual Review of Ecology, Evolution, and Systematics 11: 41–65.

Reymond P (2013) Perception, signaling and molecular basis of oviposition-mediated plant responses. Planta 238: 247–258.

Reymond P, Bodenhausen N, Van Poecke RMP, Krishnamurthy V, Dicke M, Farmer EE (2004) A conserved transcript pattern in response to a specialist and a generalist herbivore. The Plant Cell 16: 3132–3147.

Rodriguez-Saona CR, Musser RO, Vogel H, Hum-Musser SM, Thaler JS (2010) Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. Journal of Chemical Ecology 36: 1043–1057.

Romero GQ, Benson WW (2005) Biotic interactions of mites, plants and leaf domatia. Current Opinion in Plant Biology 8: 436–440.

Sanders D, Sutter L, Van Veen FJF (2013) The loss of indirect interactions leads to cascading extinctions of carnivores. Ecology Letters 16: 664–669.

Schoonhoven LM, Van Loon JJA, Dicke M (2005) Insect-Plant Biology, 2nd edn. Oxford University Press, Oxford, UK.

Schweitzer JA, Madritch MD, Bailey JK, LeRoy CJ, Fischer DG, Rehill BJ, Lindroth RL, Hagerman AE, Wooley SC, Hart SC, Whitham TG (2008) From genes to ecosystems: the genetic basis of condensed tannins and their role in nutrient regulation in a *Populus* model system. Ecosystems 11: 1005–1020.

Scriber JM, Slansky F (1981) The nutritional ecology of immature insects. Annual Review of Entomology 26: 183–211.

Shiojiri K, Takabayashi J, Yano S, Takafuji A (2001) Infochemically mediated tritrophic interaction webs on cabbage plants. Population Ecology 43: 23–29.

Smith DS, Bailey JK, Shuster SM, Whitham TG (2011) A geographic mosaic of trophic interactions and selection: trees, aphids and birds. Journal of Evolutionary Biology 24: 422–429.

Snoeren TAL, Mumm R, Poelman EH, Yang Y, Pichersky E, Dicke M (2010) The herbivore-induced plant volatile methyl salicylate negatively affects attraction of the parasitoid *Diadegma semiclausum*. Journal of Chemical Ecology 36: 479–489.

Soler R, Erb M, Kaplan I (2013) Long distance root-shoot signalling in plant-insect community interactions. Trends in Plant Science 18: 149–156.

Soler R, Ruben Badenes-Pérez F, Broekgaarden C, Zheng S-J, David A, Boland W, Dicke M (2012) Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. Functional Ecology 26: 156–166.

Staswick PE, Tiryaki I (2004) The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. The Plant Cell 16: 2117–1227.

Stireman JO, Nason JD, Heard SB, Seehawer JM (2006) Cascading host-associated genetic differentiation in parasitoids of phytophagous insects. Proceedings of the Royal Society of London B 273: 523–530.

Tack AJM, Dicke M (2013) Plant pathogens structure arthropod communities across multiple spatial and temporal scales. Functional Ecology 27: 633–645.

Thaler JS (1999) Jasmonate-inducible plant defences cause increased parasitism of herbivores. Nature 399: 686–688.

Thaler JS (2002) Effect of jasmonate-induced plant responses on the natural enemies of herbivores. Journal of Animal Ecology 71: 141–150.

Thaler JS, Fidantsef AL, Bostock RM (2002) Antagonism between jasmonate- and salicylate-mediated induced plant resistance: effects of concentration and timing of elicitation on defense-related proteins, herbivore, and pathogen performance in tomato. Journal of Chemical Ecology 28: 1131–1159.

Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science 17: 260–270.

Thaler JS, McArt SH, Kaplan I (2012) Compensatory mechanisms for ameliorating the fundamental trade-off between predator avoidance and foraging. Proceedings of the National Academy of Sciences USA 109: 12075–12080.

Thaler JS, Stout MJ, Karban R, Duffey SS (2001) Jasmonate-mediated induced plant resistance affects a community of herbivores. Ecological Entomology 26: 312–324.

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, Yang He S, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCF(COI1) complex during jasmonate signalling. Nature 448: 661–665.

Tooker JF, De Moraes CM (2008) Gall insects and indirect plant defenses: a case of active manipulation? Plant Signaling & Behavior 3: 503–504.

Underwood N (2012) When herbivores come back: effects of repeated damage on induced resistance. Functional Ecology 26: 1441–1449.

Utsumi S, Ando Y, Miki T (2010) Linkages among trait-mediated indirect effects: a new framework for the indirect interaction web. Population Ecology 52: 485–497.

Utsumi S, Ohgushi T (2008) Host plant variation in plant-mediated indirect effects: moth boring-induced susceptibility of willows to a specialist leaf beetle. Ecological Entomology 33: 250–260.

Van Veen FJF, Morris RJ, Godfray HCJ (2006) Apparent competition, quantitative food webs, and the structure of phytophagous insect communities. Annual Review of Entomology 51: 187–208.

Van Veen FJF, Van Holland PD, Godfray HCJ (2005) Stable coexistence in insect communities due to density- and trait-mediated indirect effects. Ecology 86: 3182–3189.

Van Zandt PA, Agrawal AA (2004) Community-wide impacts of herbivore-induced plant responses in milkweed (*Asclepias syriaca*). Ecology 85: 2616–2629.

Van Zandt PA, Agrawal AA (2004) Specificity of induced plant responses to specialist herbivores of the common milkweed *Asclepias syriaca*. Oikos 104: 401–109.

Viswanathan DV, Lifchits OA, Thaler JS (2007) Consequences of sequential attack for resistance to herbivores when plants have specific induced responses. Oikos 116: 1389–1399.

Viswanathan DV, Narwani AJT, Thaler JS (2005) Specificity in induced plant responses shapes patterns of herbivore occurrence on *Solanum dulcamara*. Ecology 86: 886–896

Voelckel C, Baldwin IT (2004) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. The Plant Journal 38: 650–663.

Vrieling K, Smit W, Van der Meijden E (1991) Tritrophic interactions between aphids (*Aphis jacobaeae* Schrank), ant species, *Tyria jacobaeae* L., and *Senecio jacobaea* L. lead to maintenance of genetic variation in pyrrolizidine alkaloid concentration. Oecologia 86: 177–182.

Wang D, Amornsiripanitch N, Dong X (2006) A genomic approach to identify regulatory nodes in the transcriptional network of systemic acquired resistance in plants. PLoS Pathology 2: e123.

Wang X, Hu L, Zhou G, Cheng J, Lou Y (2011) Salicylic acid and ethylene signaling pathways are involved in production of rice trypsin proteinase inhibitors induced by the leaf folder *Cnaphalocrocis medinalis* (Guenée). Chinese Science Bulletin 56: 2351–2358.

Webster B, Bruce T, Pickett J, Hardie J (2010) Volatiles functioning as host cues in a blend become nonhost cues when presented alone to the black bean aphid. Animal Behaviour 79: 451–457.

Whitham TG, Bailey JK, Schweitzer JA, Shuster SM, Bangert RK, LeRoy CJ, Lonsdorf EV, Allan GJ, DiFazio SP, Potts BM, Fischer DG, Gehring CA, Lindroth RL, Marks JC, Hart SC, Wimp GM, Wooley SC (2006) A framework for community and ecosystem genetics: from genes to ecosystems. Nature Reviews Genetics 7: 510–523.

Whitham TG, Gehring CA, Lamit LJ, Wojtowicz T, Evans LM, Keith AR, Smith DS (2012) Community specificity: life and afterlife effects of genes. Trends in Plant Science 17: 271–81.

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature 414: 562–565.

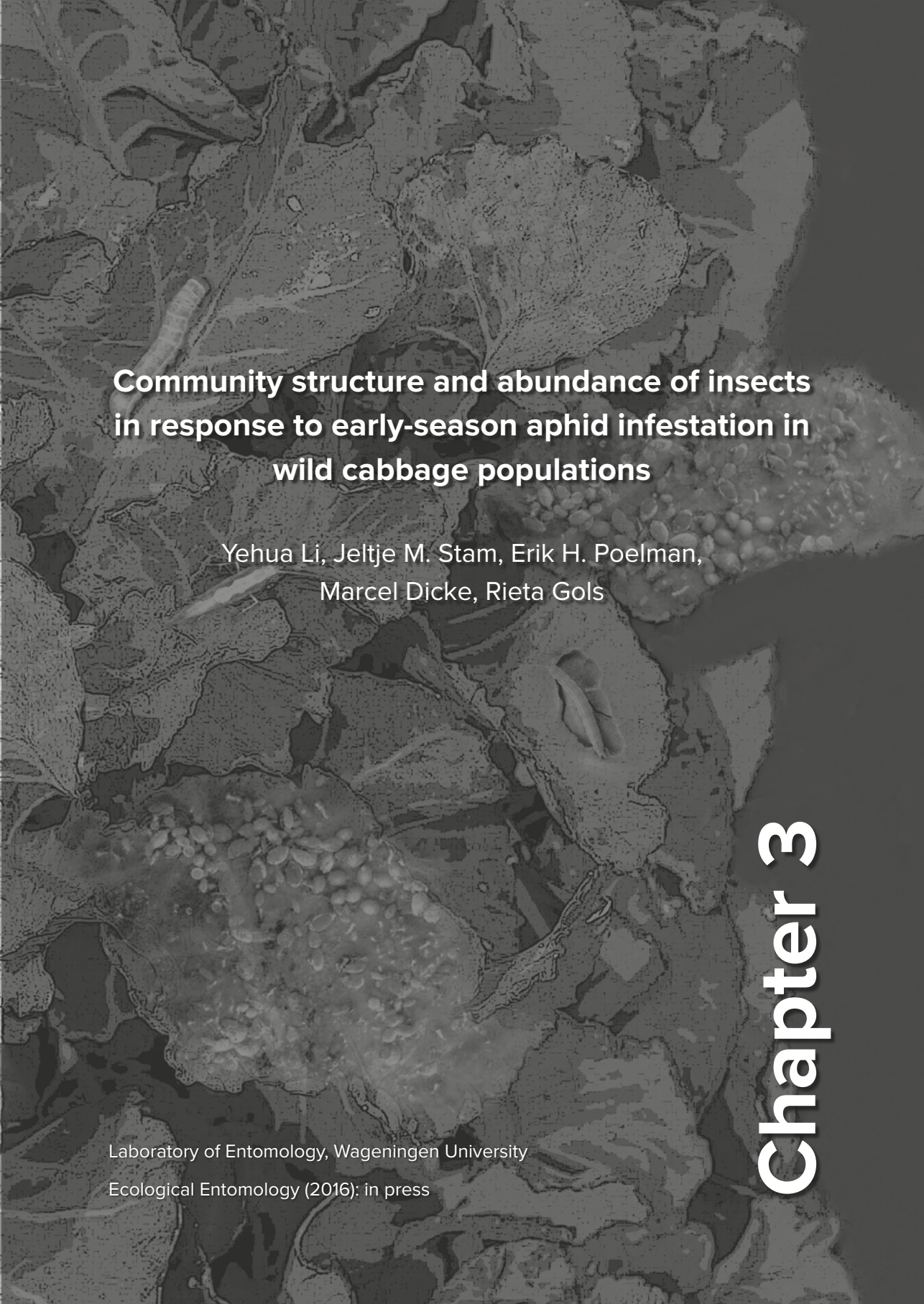
Wu JQ, Baldwin IT (2010) New insights into plant responses to the attack from insect herbivores. Annual Review of Genetics 44: 1–24.

Xiao Y, Wang Q, Erb M, Turlings TCJ, Ge L, Hu L, Li J, Han X, Zhang T, Lu J, Zhang G, Lou Y (2012) Specific herbivore-induced volatiles defend plants and determine insect community composition in the field. Ecology Letters 15: 1130–1139.

Zarate SI, Kempema LA, Walling LL (2007) Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiology 143: 866–875.

Zhang PJ, Broekgaarden C, Zheng SJ, Snoeren TAL, Van Loon JJA, Gols R, Dicke M (2013) Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. New Phytologist 197: 1291–1299.

Zhang PJ, Zheng SJ, Van Loon JJA, Boland W, David A, Mumm R, Dicke M (2009) Whiteflies interfere with indirect plant defense against spider mites in Lima bean. Proceedings of the National Academy of Sciences USA 106: 21202–21207.



# Community structure and abundance of insects in response to early-season aphid infestation in wild cabbage populations

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## Abstract

Changes in arthropod community structure can be attributed to differences in constitutively expressed plant traits or those that change depending on environmental conditions such as herbivory. Early-season herbivory may have community-wide effects on successive insect colonization of host plants and the identity of the initially inducing insect may determine the direction and strength of effects on the dynamics and composition of the associated insect community. Previous studies have addressed the effect of early infestation with a chewing herbivore. Here, we investigated the effect of early infestation with a phloem-feeding aphid (*Brevicoryne brassicae*) on the insect community associated with three wild cabbage (*Brassica oleracea*) populations, which are known to differ in defence chemistry, throughout the season in field experiments. Aphid infestation had asymmetric effects on the associated insect community and only influenced the abundance of the natural enemies of aphids, and not that of chewing herbivores and their natural enemies. The effect size of aphid infestation further depended on cabbage population. Aphid feeding has been previously reported to promote host-plant quality for chewing herbivores, which has been attributed to antagonism between the two major defence signalling pathways controlled by the hormones salicylic acid (SA) and jasmonic acid (JA), respectively. Our results show no effects of early infestation by aphids on chewing herbivores, suggesting the absence of long-term JA-SA antagonism. Investigating the effects of identity of early-season colonizer and genotypic variation among plant populations on insect community dynamics are important to understand insect-plant community ecology.

**Keywords:** aphid infestation, *Brassica oleracea*, early-season herbivory, genotypic variation, insect community, phenotypic plasticity

## Introduction

Plants serve as central players in structuring interactions among higher trophic-level organisms (Kessler and Halitschke, 2007). Changes in community structure and composition can be attributed to differences in plant traits that are constitutively expressed. For example, plant individuals with similar traits tend to support similar insect communities (Whitham et al., 2003; Whitham et al., 2006). Phenotypic plasticity, i.e. the ability to express different phenotypes depending on the biotic or abiotic environment, further contributes to variation in plant resistance traits (Agrawal, 2001; Whitham et al., 2006). The evidence for induced plant phenotypic changes in response to herbivory affecting the performance and colonization of subsequent herbivores is rapidly accumulating (Van Zandt and Agrawal, 2004; Poelman et al., 2010; Utsumi et al., 2010; Li et al., 2014). An herbivore arriving early in the growing season may affect colonization of subsequent herbivores not only directly by altering plant suitability, but also indirectly by changing interactions among colonists (Van Zandt and Agrawal, 2004; Viswanathan et al., 2005; Poelman et al., 2008; Utsumi et al., 2010).



The specificity of herbivore-induced plant responses depends on both the type of physical damage and the secreted chemical elicitors present in insect oral secretions (Kessler and Halitschke, 2007; Pieterse et al., 2009). More general, the feeding guild of the inducing herbivore is considered an important factor that determines the specificity of the induced plant response, and consequently, the direction and strength of its effect on the other community members (Van Zandt and Agrawal, 2004; Agrawal and Heil, 2012; Stam et al., 2014). At the molecular level, specificity in plant responses to herbivory can, to a large extent, be explained by differential activation of two major defence signal-transduction pathways controlled by the phytohormones jasmonic acid (JA) and salicylic acid (SA), respectively. With exceptions, phloem-feeding insects such as aphids, activate the SA-regulated defence signalling pathway, whereas biting-chewing herbivores, such as larval stages of Lepidoptera, activate the JA signalling pathway (Pieterse et al., 2009; Erb et al., 2012; Thaler et al., 2012). Moreover, these two signal-transduction pathways do not operate independently, but interact through cross-talk. For example, activation of SA signalling has been demonstrated to inhibit JA signalling and vice versa (Pieterse et al., 2009; Erb et al., 2012).

The time lag between and the sequence of different herbivory events may further influence the dynamics and direction of the plant's response (Mouttet et al., 2013). Not only are the induced plant responses herbivore specific, but also the responses of the insects exposed to the induced changes in plant phenotype can be specific and often depend on the level of dietary specialisation of these herbivores (Van Zandt and Agrawal, 2004; Kessler and Halitschke, 2007; Poelman et al., 2010; Mouttet et al., 2011; Stam et al., 2014). Herbivore-induced plant responses can also affect natural enemies of herbivorous arthropods either

directly by changes in the production or composition of plant volatile blends that serve as host / prey location cues and the production of alternative food sources such as nectar and pollen, or indirectly via plant-mediated effects on the quality and quantity of herbivorous prey or hosts (Ohgushi, 2005; Ode, 2006; Dicke and Baldwin, 2010). Moreover, the effects of induced plant responses on carnivores result in feedback effects on the herbivore community (Hunter and McNeil, 1997; Stam et al., 2014).

Herbivore-induced effects on the arthropod community have primarily been studied for situations where the initial attacker is a chewing herbivore (examples above). Though the effects of changes in aphid community composition and structure are well studied for aphid food webs (Bukovinszky et al., 2008; Finke and Snyder, 2008), less is known about how aphid infestation influences the composition of the whole community. A recent laboratory study showed that infestation by *Brevicoryne brassicae* enhanced the performance of a specialist chewing herbivore *Plutella xylostella* and its parasitoid *Diadegma semiclausum*, but did not affect the performance of a generalist chewing herbivore, *Mamestra brassicae,* and its parasitoid *Microplitis mediator* (Li et al., 2014). In the present study we have examined the effect of early aphid infestation on the arthropod community dynamics under field conditions. We used plants from three wild cabbage populations that display variation in both constitutive and herbivore-inducible foliar glucosinolate profiles (Mithen et al., 1995; Gols et al., 2008; Newton et al., 2009). Glucosinolates are secondary metabolites characteristic of the Brassicaceae (Fahey et al., 2001) and have been shown to play an important role in insect resistance (Gols and Harvey, 2008; Hopkins et al., 2009).

We asked the following questions: does 1) early-season infestation by an aphid and 2) cabbage population affect the abundance of species and community composition of associated arthropods? In a field experiment, we monitored the herbivore and carnivore community on plants originating from three wild cabbage populations that were either inoculated with the aphid *B. brassicae* L. (Hemiptera, Aphididae) or that were not inoculated with aphids early in the growing season. *Brevicoryne brassicae* is specialised on brassicaceous plants (Hughes, 1963) and is a common species on wild cabbage in its native habitat (Newton et al., 2009). In addition, we monitored parasitism of one of the most abundant chewing herbivores *Plutella xylostella* L. (Lepidoptera: Plutellidae) in detail by releasing and re-capturing larvae of this species and determine whether they were parasitized or not.

Previous studies have shown that infestation by aphids can promote the performance of chewing herbivores through negative interaction between the JA and SA defence signalling pathways (Zarate et al., 2007; Rodriguez-Saona et al., 2010; Soler et al., 2012). Assuming antagonism between SA and JA-regulated induced defence responses, initial infestation with a phloem-feeding aphid early in the season is hypothesized to inhibit JA-related defence responses, which in turn will benefit the performance of chewing herbivores and their associated natural enemies resulting in increased abundance of these insects.

## Materials And Method

### Plants and insects

Seeds from three wild cabbage populations growing naturally along the south-coast of England, were collected in Dorset, U.K., at sites known as Kimmeridge (50°36'N, 2°07'W), Old Harry (OH; 50°38'N, 1°55'W), and Winspit (WIN; 50°35'N, 2°02'W), hereafter KIM, OH and WIN, respectively. *Plutella xylostella* is a specialist biting-chewing herbivore on brassicaceous plant species, and, like *B. brassicae*, a destructive pest on cabbage crops (Furlong et al., 2012) and was used to determine levels of parasitism (described below). *Plutella xylostella* larvae and *B. brassicae* that were used for infestation of plants were obtained from laboratory cultures. Both insect cultures were maintained on Brussels sprouts (*B. oleracea* L. var gemmifera cv. Cyrus) plants in a greenhouse compartment or a climate room at 22 ± 2˚C, 60-70% RH and 16:8 h L:D photo regime.



### Common garden set-up and aphid treatment

Seeds of the three cabbage populations were germinated on peat soil (Lentse potgrond BV, Lent, The Netherlands), and one-week-old seedlings were transplanted into peat soil cubes. Soil cubes with two-weeks-old seedlings were placed outside to acclimatize to field conditions for one week, before they were transplanted into the experimental field in week 21 of 2012 and 2013.

The experimental design for year 2012 consisted of 32, 4-by-4 m plots which were planted with monocultures of either 12 KIM (16 plots) or 12 WIN (16 plots) plants that were arranged in a square with no plants in the centre. For the experiment in 2013, forty-eight plots (7 × 7 m) were established, each containing monocultures of 36 plants of one of the three wild cabbage populations, KIM, WIN or OH, respectively. Cabbage populations were assigned to the plots according to a randomized design. In each plot, plants were planted one meter apart in bare soil (weeding was performed regularly). A 4-m-wide strip sown with *Lolium* and *Poa* grasses separated the plots. Along the borders of the experimental fields, two rows of *Brassica nigra* plants were planted (0.5 m between plants within a row; 1 m between rows), to create a uniform vegetation around the field. A wide-meshed plastic fence surrounded the fields in both years to prevent feeding by rabbits and hares. One (2012) or two weeks (2013) after establishment of the plants in the field, all plants in half of the randomly selected plots of each cabbage population received five *B. brassicae* in a mixture of adults and 4th instar nymphs, onto the first fully expanded leaf.

### Insect community monitoring

In 2012, from week 23 (early June) until week 40 (early October), naturally occurring insects were monitored every week in the early season and every two weeks as the season progressed. Four plants in each plot were randomly selected and monitored consistently at all monitoring times. In 2013, from week 24 (early June) until week 39 (late September), insects on the four central plants of each plot were monitored every two weeks (even weeks). All insects were counted on both leaf sides and their identity was determined (for a species list see Table 1). Insects of the same species in different stages of development found on one plant were pooled. Aphids were recorded as parasitized when they were mummified. As aphids on plants reached sometimes very high numbers (maximum around 3000 individuals per plant), we estimated the number of aphids by counting groups of approximately 20 individuals. In addition, the number of leaves, plant height and the score of foliar damage were recorded for the four central plants in each plot in 2013. Foliar damage was visually estimated and scored at a linear scale from 0 (=no damage) to 5 (=50 % of the leaves were consumed).



### Assessment of parasitism of *Plutella xylostella*

Parasitism of *P. xylostella* caterpillars was determined from week 25 (early June) until week 33 (middle of August) in 2013 when *P. xylostella* was abundant. Every two weeks (odd weeks), one of the 12 plants neighbouring the four central plants in each plot was randomly selected for infestation with *P. xylostella* caterpillars. A small piece of Brussels sprouts leaf infested with 20 second-instar caterpillars was attached onto a young leaf of the selected plant using a paperclip. The caterpillars were re-collected three days after introduction into the field. As we could not distinguish the inoculated caterpillars from the ones that occurred naturally, all *P. xylostella* caterpillars were collected from the selected plant and reared in the laboratory on Brussels sprout leaves until a moth or a wasp eclosed. All eclosed insects were counted and identified based on their morphology. The most common parasitoid wasp species were *Diadegma semiclausum*, *Diadegma fenestralis*, *Cotesia vestalis*, and *Dolichogenidea sicaria*. Some rare wasp species and hyperparasitoids that were collected were not identified further. The parasitism ratio of *P. xylostella* was determined as the fraction of the number of wasps eclosing from the total number of collected caterpillars. The data generated for measurement of parasitism were not included in the data set on the insect community described in the previous section and were analysed separately.

### Data analysis

To determine whether the insect community was affected by *B. brassicae* infestation and whether this effect differed among the three cabbage populations, we constructed principle response curves (PRC) using the CANOCO software package 5 (ter Braak

and Šmilauer, 2012). This multivariate method, allows for comparison of community composition in experimental designs sampled repeatedly in time (Poelman et al., 2010). As fixed factors, we entered cabbage population and aphid treatment in the statistical model, while sampling week was entered as a co-variate. For each monitoring week, we averaged per plot the number of insect individuals counted on the four plants in the centre of the plots. Counts were log(x+1)-transformed because high species counts tend to influence the PRC analysis more strongly than low ones.

**Table. 1** Common insect species found on three wild *Brassica oleracea* populations grown in a field experiment in the Netherlands in 2012 and 2013.

| Common name | Species | Order | Family | Feeding type | Host specificity |
|---|---|---|---|---|---|
| Cabbage flea beetle | *Phyllotreta undula* | Coleoptera | Chrysomelidae | Leaf chewer | Specialist |
| Black flea beetle | *Phyllotreta atra* | Coleoptera | Chrysomelidae | Leaf chewer | Specialist |
| Western tarnished plant bug | *Lygus hesperus* | Hemiptera | Miridae | Phloem feeder | Generalist |
| Cabbage whitefly | *Aleyrodes proletella* | Hemiptera | Aleyrodidae | Phloem feeder | Specialist |
| Cabbage aphid | *Brevicoryne brassicae* | Hemiptera | Aphididae | Phloem feeder | Specialist |
| Black bean aphid | *Aphis fabae* | Hemiptera | Aphididae | Phloem feeder | Generalist |
| Green peach aphid | *Myzus persicae* | Hemiptera | Aphididae | Phloem feeder | Generalist |
| Diamondback moth | *Plutella xylostella* | Lepidoptera | Plutellidae | Leaf chewer | Specialist |
| Cabbage moth | *Mamestra brassicae* | Lepidoptera | Noctuidae | Leaf chewer | Generalist |
| Large cabbage white | *Pieris brassicae* | Lepidoptera | Pieridade | Leaf chewer | Specialist |
| Small cabbage white | *Pieris rapae* | Lepidoptera | Pieridade | Leaf chewer | Specialist |
| Dog's tooth | *Lacanobia suasa* | Lepidoptera | Noctuidae | Leaf chewer | Generalist |
| Silver Y | *Autographa gamma* | Lepidoptera | Noctuidae | Leaf chewer | Generalist |
| Garden pebble | *Evergestis forficalis* | Lepidoptera | Crambidae | Leaf chewer | Specialist |
| Onion thrips | *Thrips tabaci* | Thysanoptera | Thripidae | Cell content feeder | Generalist |
| Hoverflies | | Diptera | Syrphidae | Aphid predator | |
| Lacewings | | Neuroptera | Chrysopidae | Aphid predator | |
| Predatory midges | | Diptera | Cecidomyidae | Aphid predator | |
| Lady beetles | | Coleoptera | Coccinellidae | Aphid predator | |
| Aphid parasitoids | | Hymenoptera | Braconidae | Parasitic wasp | |
| | *Cotesia glomerata* | Hymenoptera | Braconidae | Parasitic wasp | Specialist |
| | *Cotesia vestalis* | Hymenoptera | Braconidae | Parasitic wasp | Specialist |
| | *Cotesia rubecula* | Hymenoptera | Braconidae | Parasitic wasp | Specialist |
| | *Diadegma semiclausum* | Hymenoptera | Ichneumonidae | Parasitic wasp | Specialist |
| | *Diadegma fenestrale* | Hymenoptera | Ichneumonidae | Parasitic wasp | Generalist |
| | *Microplitis mediator* | Hymenoptera | Braconidae | Parasitic wasp | Generalist |

The PRC method uses partial redundancy analysis (RDA) and plots the first principal component against time and at the same time contrasts each treatment (6 in total, three populations each with or without initial aphid infestation treatment) against a pre-set baseline. As we were interested in the effect of early aphid infestation, it makes sense to use one of the plant populations that have not been exposed to aphids and also use a population that has been used in both years. We arbitrarily have chosen KIM control as the baseline treatment. The multivariate counterpart of the ordinary *F*-ratio in univariate statistics was calculated using the sums of squares totalled across all response variables (here species counts) to yield the $H_0$ F-statistic. Monte Carlo permutation (default setting of 499 permutations) tests were used to determine whether models were significant or not (Lepš and Šmilauer, 2003).

We progressively reduced the data set and constructed additional PRC curves to investigate the effect of aphid infestation and plant intraspecific variation on subsets of the community which is described in detail in the Results section. When the PRC analysis revealed significant effects of plant population or aphid treatment on specific insect groups, pairwise comparisons were conducted to test for significant differences between treatment groups.



We tested for differences in the numbers of *P. xylostella* as well as the fraction of *P. xylostella* hosts that were parasitized over time using Generalized Linear Mixed Models (GLMMs). Cabbage population, aphid treatment, monitoring weeks as well as their interaction were entered as fixed factors in the model, whereas plot number was entered as a random factor. Similarly, measurements of plant morphological traits (foliar damage score, plant height and leaf numbers) in 2013, were analysed using GLMMs with the same model terms. When the model was significant, pair-wise comparisons among the means were performed using Tukey-Kramer tests. We used the statistical packages Genstat (16th edition, VSN International, UK) and SAS version 9.3 (SAS Institute Inc, Cary, NC, USA).

## Results

### Whole insect community

The majority of the species (Table 1) were observed in both years, on all cabbage populations and on both aphid-inoculated and aphid-free plants. PRC analysis using the complete community data set revealed overall significant differences among the six population-treatment combinations both in 2012 and 2013 (Fig 1). In both years (Fig 1), *B. brassicae* and aphid mummies (indicated by their high species weights) contributed the most to the statistical differentiation of the insect community. Species scores together with PRC scores can be used to compare the abundance of a species in a treatment group relative

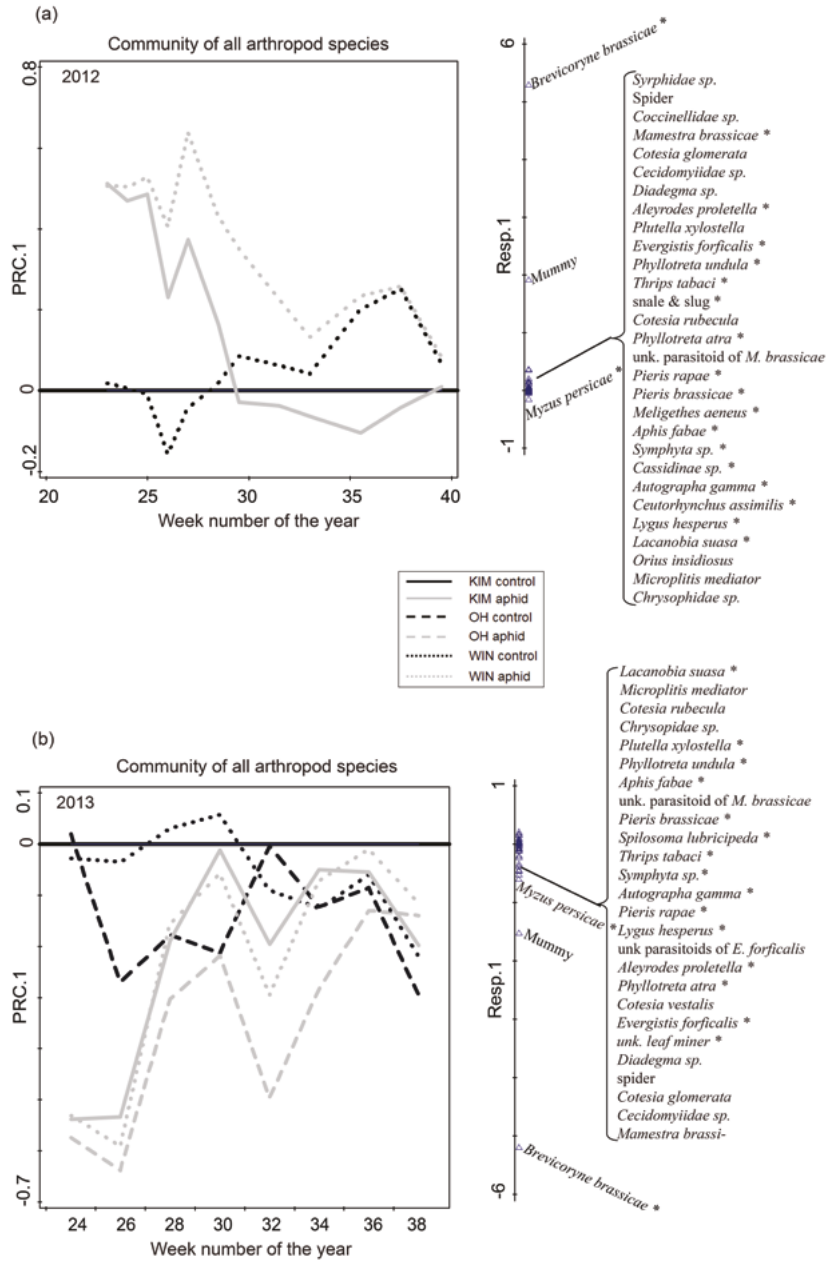



**Figure 1.** Principal response curves (PRC) for the abundance of all insect species throughout the season [week 23-39 in 2012 (a); week 24-38 in 2013 (b)] on plants from wild cabbage (*Brassica oleracea)* populations [KIM and WIN in 2012; KIM, OH and WIN in 2013] that were (grey lines) or were not (black) infested with *Brevicoryne brassicae* aphids early in the season (week 22 in 2012; week 23 in 2013). Solid lines refer to the KIM, dashed lines to the OH and dotted lines to the WIN population. The KIM control group was arbitrarily set as the baseline against which the other groups were contrasted. Species weights (the abundances of the species taken over all the samples) on the first principal component are depicted in a score plot on the right side of the figure. Species names marked with an asterisk are herbivore species. The statistics (explained variance, *F*-test results based on Monte Carlo permutation tests) for the depicted first PRC-axes are: (a) 13.9%, $F$=54.1, $P$=0.002; (b) 7.54%, $F$=27.4, $P$=0.002).

to the pre-set base line (here KIM plants not exposed to aphids). The base of the natural logarithm, e, raised to the power of the PRC value of a specific curve at a given time point multiplied by a species score, gives the relative abundance of that species compared to the control at the same time point (Lepš and Šmilauer, 2003). For example, in week 27 (Fig 1a), the average abundance of *B. brassicae* was approximately 31 (=EXP(0.65*5.3)) times higher in the WIN population infested with aphids (WIN aphid), than in KIM plants that were not exposed to aphids (KIM control), whereas on WIN control plants (WIN control) the relative abundance of *B. brassicae* was on average 0.9 (=EXP(-0.025 *5.3)) times the abundance found on KIM control plants. The models also showed that the effect of aphid infestation waned when the season progressed (Fig 1).

To investigate how insect species other than *B. brassicae* responded to the early-season aphid infestation, PRC analyses were performed on the complete data set excluding *B. brassicae*. The PRC model for 2012 showed significant differences among the population-treatment combinations ($F$= 15.1, $P$= 0.01), whereas they were not significant for 2013 ($F$= 9.2, $P$= 0.30). The difference between these two years appeared to be caused by the fact that the effect of the aphid infestation waned faster in 2013 than in 2012. To test this, we decided to analyse the data of 2013 separately for the first half (weeks 24-30) and the second half of the experimental period (weeks 32-38). Indeed, the PRC models were not significant for either the herbivore or the natural enemy community in the second half of the season in 2013 (Appendix 1 and 2). The data of 2012 were analysed over the entire experimental period only, whereas we used only the dataset obtained for the first half of the experimental period in the analyses described below.



**Herbivore community**

Over the entire experimental period (weeks 23-39) in 2012, the herbivore community excluding *B. brassicae* was similar on plants from the KIM and WIN population, and was not affected by early-season aphid infestation (Fig 2a; effect of plant population $F$=7.2, $P$=0.19; effect of *B. brassicae*-infestation $F$=53, $P$=0.49). Considering only the aphid community (*B. brassicae*, *M. persicae*, *A. fabae*) resulted in a model that was significantly affected by the early-season *B. brassicae*-infestation, and marginally by plant population (Fig 2b; effect of *B. brassicae*-infestation, $F$=165, $P$=0.002; effect of plant population, $F$=20.1, $P$=0.056). This results shows that introduction of *B. brassicae* aphids was successful, but also that other herbivorous community members did not respond to the introduction of this aphid.

Similar to 2012, PRC analysis of the herbivore community, excluding *B. brassicae*, during the first half of the season (week 24-30) in 2013 showed that aphid-infestation treatment had no effect on the herbivore community ($F$=2.3, $P$=0.40, Fig 2c). Infestation with *B. brassicae* only increased the abundance of this aphid species irrespective of cabbage population (PRC analysis of only aphids: effect of *B. brassicae*-infestation, $F$=102, $P$=0.002;

effect of plant population $F$=10.3, $P$=0.13; Fig 2d). However, the herbivore community differed among the plant populations ($F$=9.63, $P$=0.008); the herbivore community on WIN plants differed from the community on OH and KIM plants (WIN-OH, $F$=6.2, $P$=0.04; WIN-KIM, $F$=6.8, $P$=0.006; KIM-OH, $F$=2.0, $P$=0.63). The species that contributed the most to the statistical separation of the treatment groups was *M. brassicae*, followed by *M. persicae*. These species were more abundant on OH and KIM than on WIN plants.

### Carnivore community

In both years, the community of carnivore species was affected by aphid-infestation treatment and also developed differently on the cabbage populations (effect of *B. brassicae*-infestation: 2012, $F$= 24.8, $P$= 0.002; 2013, $F$=7.0, $P$=0.01; effect of plant population: 2012, $F$=13.6, $P$=0.01; 2013, $F$=7.8, $P$=0.02; Fig 3a, 3b). Aphid infestation primarily affected aphid parasitoids and to a lesser extent other aphid natural enemies (syrphid, coccinellid and cecidomyiid species). Separate PRC analyses of aphid and non-aphid carnivore communities, respectively, confirmed this. In both years, aphid infestation had a significant effect on carnivores of aphids (2012, $F$=45.4, $P$=0.002; 2013, $F$=11.7, $P$=0.008) but not on the communities of the other carnivores (2012, $F$=8.9, $P$=0.16, 2013, $F$=1.4, $P$=0.73). In both years, plant population also affected the community of aphid carnivores (2012, $F$=21.4, $P$=0.006; 2013, $F$=13.5, $P$=0.004), whereas the composition of the non-aphid carnivore community was not affected by cabbage population (2012, $F$=6.6, $P$=0.34; 2013, $F$=9.9, $P$=0.08). Early-season infestation with *B. brassicae* affected the abundance of aphid carnivores on WIN and OH plants (OH was tested in 2013 only) more strongly than on KIM plants (WIN control vs. WIN aphid: 2012, $F$=42.5, $P$=0.004; 2013, $F$=7.5, $P$=0.024; OH control vs. OH aphid: 2013, $F$=9.0, $P$=0.006; KIM control, vs. KIM aphid: 2012, $F$=12, $P$=0.052; 2013, $F$=1.0, $P$=0.98, Fig 3b, 3d). Aphid parasitoids (mummies) contributed most to the statistical separation of the communities on control and *B. brassicae*-infested plants.

### Plant morphological traits in 2013

Analysis of the model incorporating seasonal effect as a co-variate showed that WIN plants developed significantly more leaves than KIM pants (Appendix 3, KIM-WIN $P$= 0.037; KIM-OH $P$= 0.15; OH-WIN $P$= 0.80). In addition, WIN plants were shorter than plants from the other two populations (KIM-WIN $P$= 0.0018; OH-WIN $P$< 0.001; KIM-OH $P$= 0.92). Damage levels did not differ among the plant populations. There was no effect of early-aphid infestation on any of the morphological traits quantified.

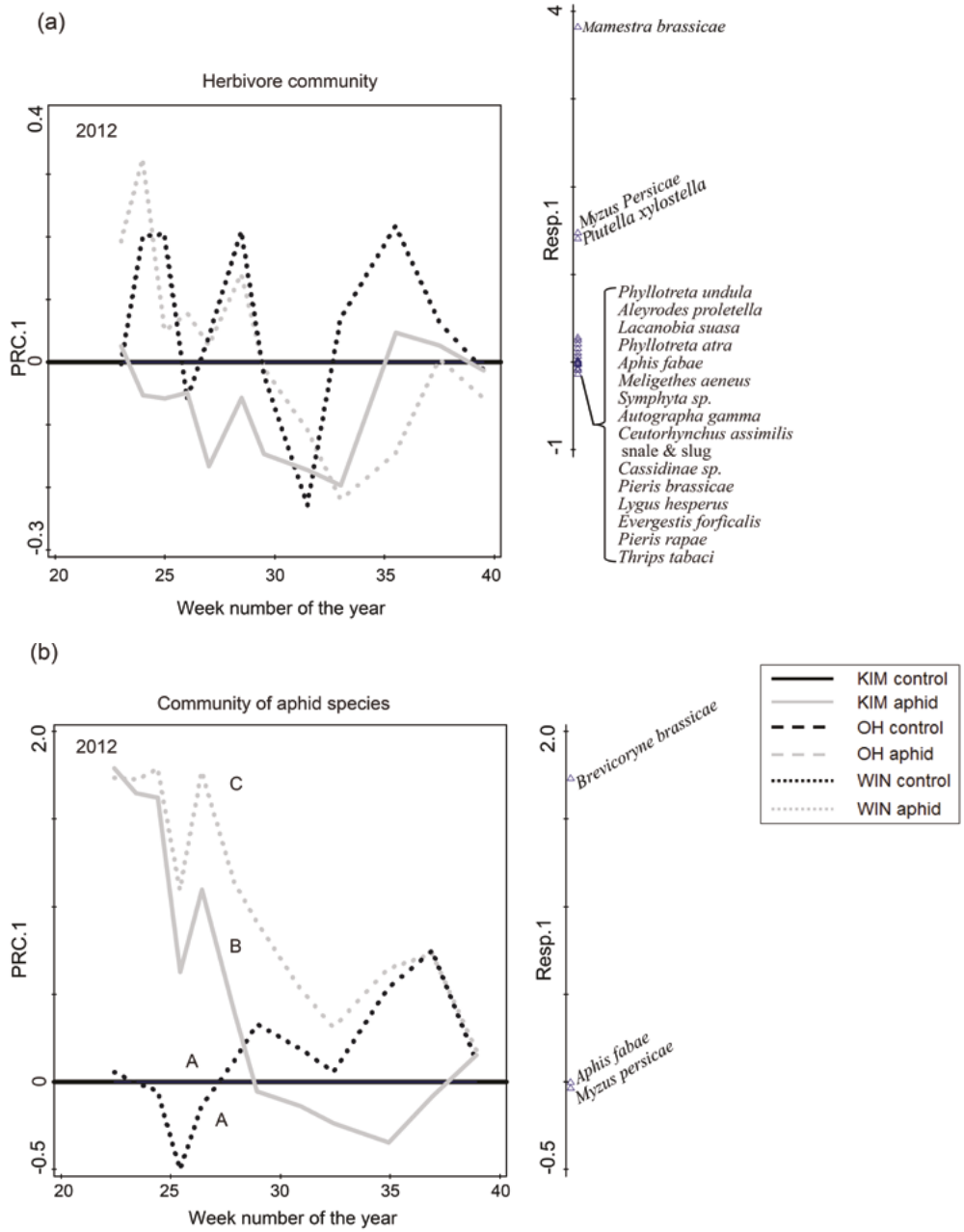
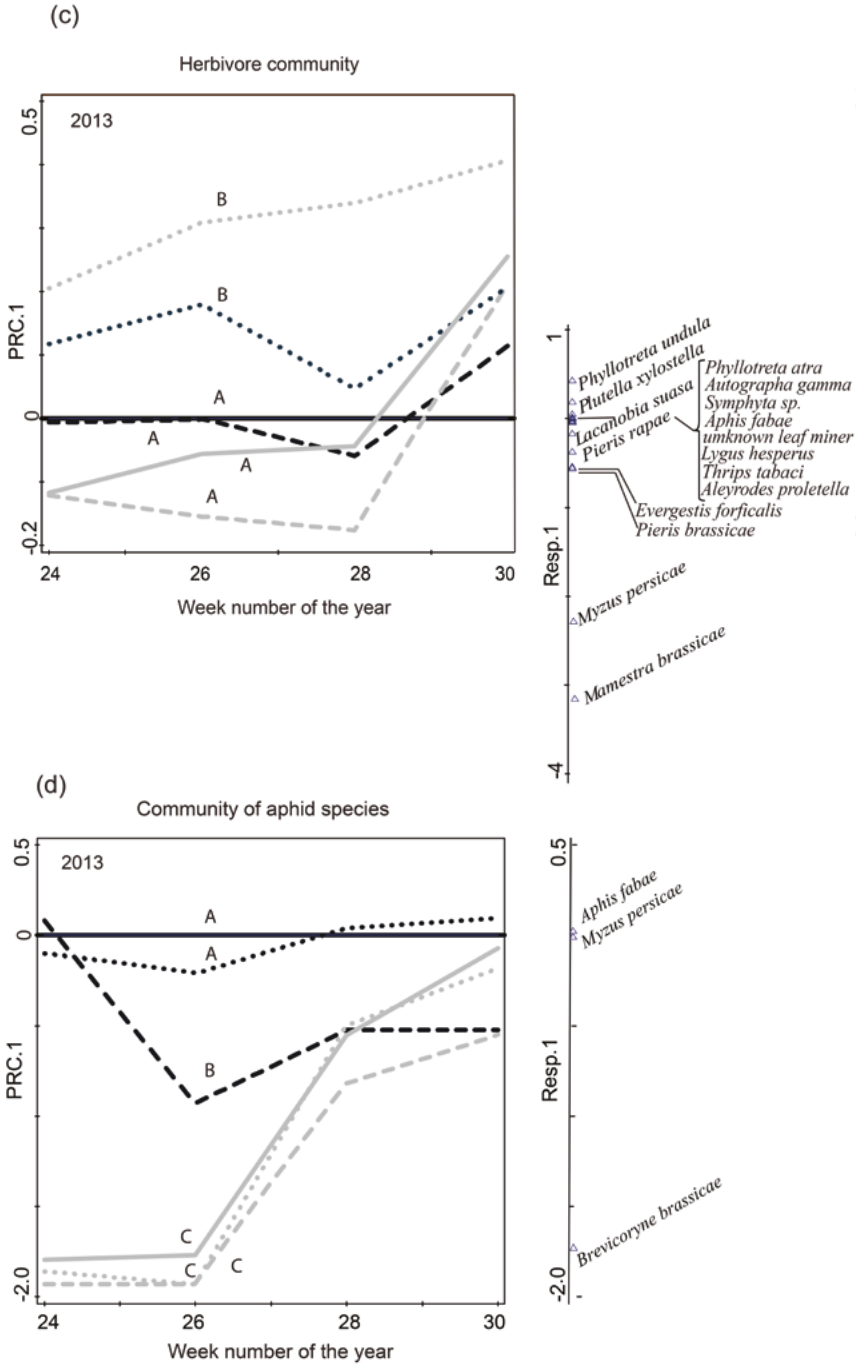



**Figure 2.** Principal response curves (PRC) of the complete herbivore community (a, c) and the aphid community (b, d) monitored throughout the growing season in (a-b) 2012 (week 23-39), and in (c-d) the first half of the growing season in 2013 (week 24-30) on plants from wild cabbage (*Brassica oleracea)* populations (KIM and WIN in 2012; KIM, OH and WIN in 2013) that were (grey lines) or were not (black lines) infested with *Brevicoryne brassicae* aphids early in the season (week 22 in 2012; week 23 in 2013). Solid lines refer to the KIM, dashed lines to the OH and dotted lines to the WIN population. The KIM control group was arbitrarily set as the baseline against which the other groups were contrasted. Species weights on the first principal component are depicted in a score plot on




the right side of the figure. The statistics (explained variance and *F*-test results based on Monte Carlo permutation tests) for the depicted first PRC-axes are: (a) 4.38%, *F*=15.4, *P*=0.34; (b) 38.6%, *F*=211, *P*=0.002; (c) 7.06%, *F*=12.8, *P*=0.016; (d) 40.1%, *F*=112, *P*=0.002). Different capital letters next to the curves indicate significant differences among the plant-treatment groups based on pairwise PRC analyses.

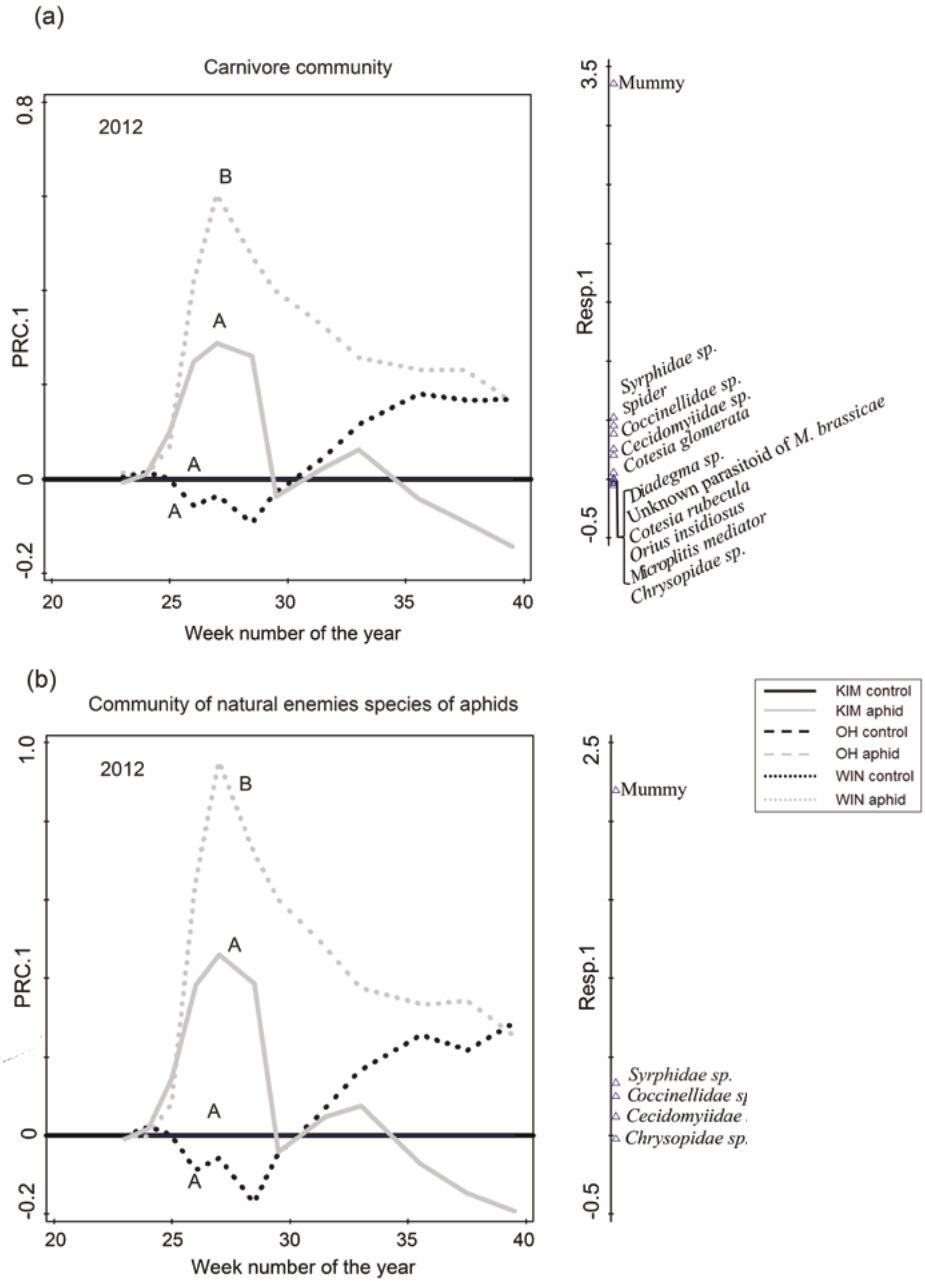
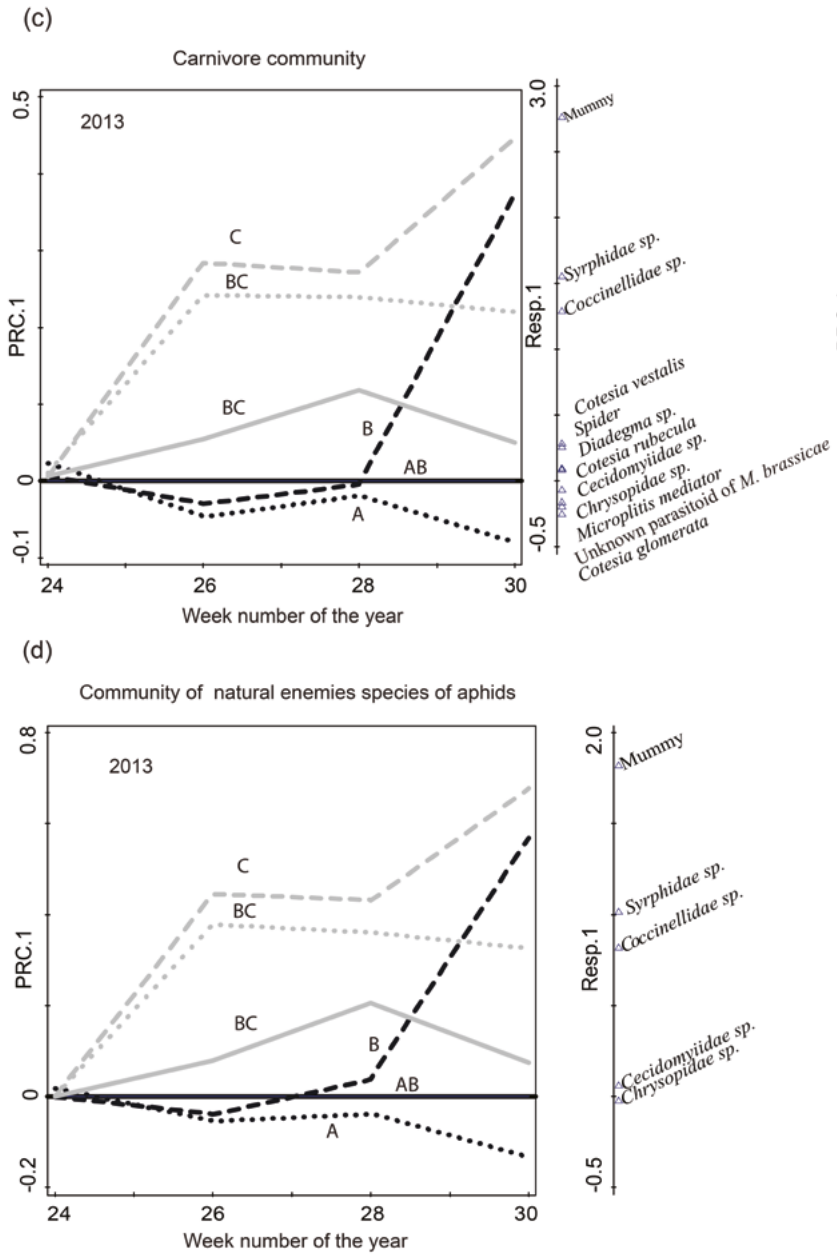



**Figure 3.** Principal response curves (PRC) of (a, c) the complete and (b, d) the aphid-associated carnivore community monitored throughout the growing season (week 23-39), and in (c-d) the first half of the season of 2013 (week 24-30) on plants from wild cabbage (*Brassica oleracea)* populations (KIM and WIN in 2012; KIM, OH and WIN in 2013) that were (grey lines) or were (black lines) not infested with *Brevicoryne brassicae* aphids early in the season (week 22 in 2012 and week 23 in 2013). Solid lines refer to the KIM, dashed lines to the OH and dotted lines to the WIN population. The KIM control group was arbitrarily set as the baseline against which the other




groups were contrasted. Species weights on the first principal component are depicted in a score plot on the right side of the figure. The statistics (explained variance, *F*-test results based on Monte Carlo permutation tests) for the depicted first PRC-axes are: (a) 10.5%, *F*=39.4, *P*=0.002; (b) 18.3%, *F*=75.4, *P*=0.002; (c) 8.24%, *F*= 5.1, *P*=0.004; (d) 14.0%, *F*=27.4, *P*=0.002). Different capital letters next to the curves indicate significant differences among the plant-treatment groups based on pairwise PRC analyses.

### Parasitism percentage of *P. xylostella* larvae

Population densities of *P. xylostella* peaked in week 26 on all cabbage populations, and then gradually declined and reached very low numbers after week 31 (Fig 4a; effect of time, $F_{7,336}$=300, $P$< 0.001). The mean number of recollected *P. xylostella* larvae was significantly higher on KIM and WIN plants than on OH plants (Fig 4a; effect of plant population, $F_{2,336}$= 3.10, $P$= 0.046), but was similar on aphid-inoculated and control plants irrespective of cabbage population (Fig 4a; effect of aphid treatment, $F_{1,336}$= 1.02, $P$= 0.31; interaction between aphid treatment and plant population, $F_{2,210}$= 0.42, $P$= 0.66).

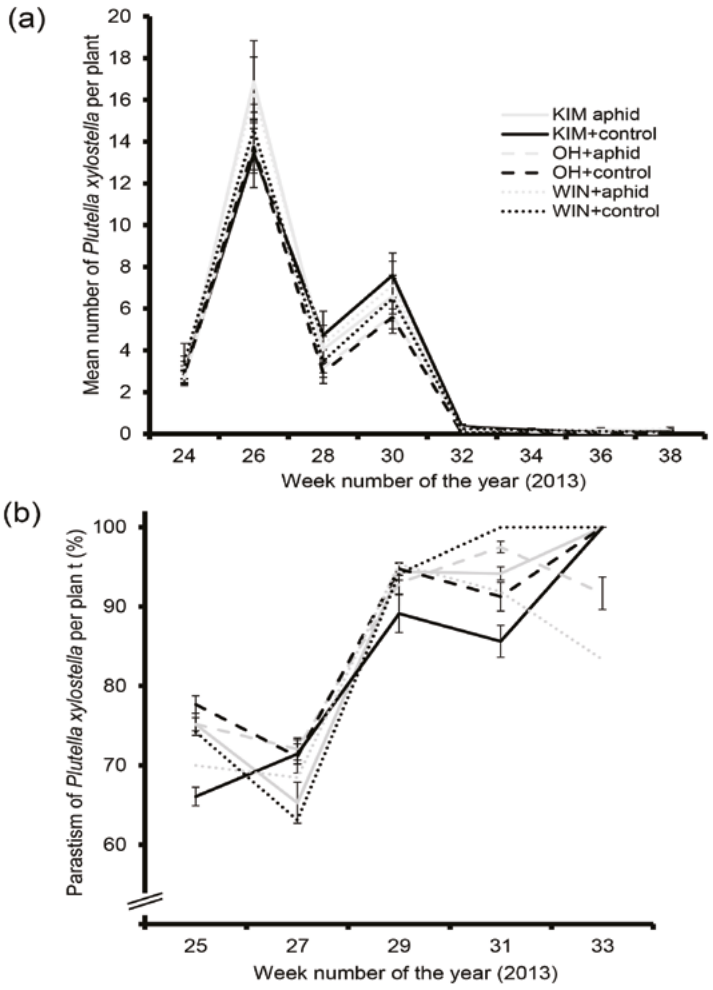



**Figure 4.** Mean abundance (a) of *Plutella xylostella* (± SE) per plant from week 24 to week 38 in 2013 and parasitism (b) (percentage parasitized caterpillars ± SE) of *P. xylostella* larvae per plant based on release-recapture experiments conducted from week 25 to week 33 in 2013 on three cabbage populations (KIM, solid lines; OH dashed lines; WIN dotted lines) either with (grey lines) or without (black lines) early aphid (*Brevicoryne brassicae*) infestation.

On all three cabbage populations, the percentage of parasitism increased over time (Fig 4b; effect of time $F_{4,199}$= 38.7, $P$< 0.001). Parasitism of *P. xylostella* larvae did not differ, neither among the cabbage populations nor between aphid-inoculated and control plants (Fig 4b; effect of plant population $F_{2,199}$= 0.29, $P$= 0.75; effect of aphid treatment $F_{1,199}$= 0.1, $P$= 0.75). For all population-treatment groups, the average percentages of parasitized caterpillars ranged between 73 and 96 % over the experimental period.

## Discussion

Early-season infestation with *B. brassicae* of plants originating from three chemically distinct wild cabbage populations only affected a subset of the associated insect community. Initial *B. brassicae* infestation significantly increased the abundance of this species, showing a clear aphid-infestation effect, and also increased the abundance of natural enemies of aphids. This pattern was consistent across the two experimental years. In addition, the effect of *B. brassicae* infestation on natural enemies of aphids differed among the three cabbage populations. Parasitism of *P. xylostella* caterpillars was similar on all three cabbage populations with or without *B. brassicae*-infestation treatment.



Most of the insect species were found on plants from all three cabbage populations irrespective of *B. brassicae*-infestation. Thus, aphid treatment only affected the abundance of various species but not the species composition of the arthropod community associated with cabbage. Moreover, the effect of early-season infestation with *B. brassicae* on the abundance of the associated arthropod community was less pronounced compared to early colonization by chewing herbivores studied previously (Rodriguez-Saona and Thaler, 2005; Poelman et al., 2010), (Van Zandt and Agrawal, 2004; Viswanathan et al., 2005; Poelman et al., 2008). Whereas chewing herbivores can cause severe damage by removing photosynthetic tissues, the impact of aphid feeding is more subtle, but can eventually also be detrimental to the plants. Aphid feeding can result in deformation of tissues especially shoot tips, transmission of plant viruses, and honeydew excretions can serve as a substrate for fungus development (Guerrieri and Digilio, 2008). Feeding by aphids is characterized by minimal tissue damage to the host plants, which is restricted to the sub-cellular level. Moreover, the induced plant phenotypic changes in response to aphid feeding are localized to the feeding site and are short-lived (Moran and Thompson, 2001; Walling, 2008; Rodriguez-Saona et al., 2010). This contrasts with the induced responses to chewing caterpillars, which are systemically expressed and long lasting (Dicke, 1994; Zeier, 2005; Poelman et al., 2010). Consequently, the effect of aphid-induced plant responses on the herbivore community may be relatively small compared to those induced by caterpillar feeding, and only influences organisms associated with aphids.

Short-term laboratory studies with chewing herbivores on plants that had been exposed to aphids reported an increased performance of chewers on aphid-infested plants compared to control plants and attributed this to the antagonism between JA- and SA-regulated induced plant responses. Here, we show that long-term studies conducted in the field on wild cabbage plants do not support JA-SA antagonism (Zarate et al., 2007; Rodriguez-Saona et al., 2010; Soler et al., 2012; Li et al., 2014) when plants are infested with *B. brassicae*. Possibly the effects of JA-SA antagonism on chewing herbivores are relatively small, affecting biomass and development time, but not survival of the herbivores. The effects of JA-SA antagonism may be statistically significant in the lab, but not ecologically significant in the field. Alternatively, JA-SA antagonism may be transient and plants may adjust their response when attacked by other herbivores arriving later in the season.

The differences in community responses to aphid infestation among the three cabbage populations were relatively small and primarily affected the natural enemies of the aphids. Few studies have quantified the influence of plant traits on the composition and abundance of the arthropod community beyond the first trophic level of consumers (Bukovinszky et al., 2008; Poelman et al., 2009). Aphid infestation early in the growing season increased the abundance of aphid natural enemies more on WIN and OH plants than on KIM plants. This result can be explained by either a direct aphid-density response or an indirect response related to changes in the plant, e.g. the enhanced production of aphid-induced carnivore attractants. Controlled for genotypic variation, Newton et al. (2009) reported a strong positive correlation between *B. brassicae* density and parasitism by *Diaeretiella rapae*, the most common parasitoid of *B. brassicae*. In the present study, there was no effect of cabbage population on the population dynamics of *B. brassicae* in either of the two years. Moreover, in the laboratory we also found no differences in population increase of *B. brassicae* on the three plant populations (Li et al. 2014). These findings indicate that there are no differences in host-plant quality for *B. brassicae* population development and, consequently, that the effects on aphid natural enemy abundance are probably indirect.

The wild cabbage populations used in this study are known to exhibit variation in secondary foliar chemistry, which differentially affects growth and development of herbivores as well as their natural enemies (Gols et al., 2008; Li et al., 2014). For example, glucosinolates in KIM plants are most inducible in response to feeding by *P. rapae* caterpillars compared to OH and WIN plants (Gols et al., 2008). In contrast, Li et al (2014) reported that infestation with *B. brassicae* facilitated growth and development of *P. xylostella,* but the effect was the least pronounced in KIM plants compared to OH and WIN. The results of the study by Li et al. (2014) and this study suggest that KIM plants are less responsive to aphid infestation than OH and WIN plants. Gols et al. (2011) reported that plants of the three cabbage populations used in this study also emit volatile blends that differ considerably when attacked by a chewing herbivore. To what extent differences in induced phytochemistry contribute to the observed differences in the abundance of primarily aphid natural enemies needs further investigation.

In addition to plant secondary chemistry, variation in plant morphological traits, such as plant architecture and plant size, has been shown to affect insect community composition, and may be more important than chemical defence traits (Johnson and Agrawal, 2005; Carmona et al., 2011). We also measured population-related variation in plant growth and found that WIN plants gained less height than KIM and OH plants but developed more leaves compared to KIM plants, and this may have contributed to the observed differences in the abundance of associated insect species.

Patterns of early-season *B. brassicae*-infestation and cabbage population on the insect community were consistent across the two experimental years, although the effects of early aphid infestation waned faster in 2013 than in 2012, which can be explained by differences in environmental conditions between the two years. Moreover, the effect of *B. brassicae*-infestation on insect community waned when the season progressed in both 2012 and 2013. Seasonal variation in insect responses to plant chemical traits was also reported for the wild cabbage populations in their natural habitat in the UK (Newton et al., 2009). Plant-mediated effects on herbivores are not constant over the growing season and can be attributed to changes in herbivore densities and chemical traits of the plants (Ohgushi, 2005; Gols et al., 2007).



In conclusion, we have shown that early infestation with *B. brassicae* only affected natural enemies associated with aphids, which can be explained by direct chain-interaction links (Mooney and Agrawal, 2008), most likely indirectly through aphid-induced changes in the plant's phenotype, but a direct density response cannot be excluded. We found no evidence for JA-SA antagonism, as the chewing herbivore community and their natural enemies were not affected by aphid infestation early in the season. Further research should reveal whether this pattern is consistent across aphid and plant species. Studying the effect of variable resistance traits in natural plant populations and environmental factors such as herbivory at a community-scale will deepen our understanding of how plant-insect communities function and are structured.

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## References


Agrawal AA (2001) Phenotypic plasticity in the interactions and evolution of species. Science 294: 321-326.

Agrawal AA, Heil M (2012) Synthesizing specificity: multiple approaches to understanding the attack and defense of plants. Trends in Plant Science 17: 239-242.

Bukovinszky T, van Veen FJF, Jongema Y, Dicke M (2008) Direct and indirect effects of resource quality on food web structure. Science 319: 804-807.

Carmona D, Lajeunesse MJ, Johnson MTJ (2011) Plant traits that predict resistance to herbivores. Functional Ecology 25: 358-367.

Dicke M (1994) Local and systemic production of volatile herbivore-induced terpenoids: their role in plant-carnivore mutualism. Journal of Plant Physiology 143: 465-472.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends in Plant Science 15: 167-175.

Erb M, Meldau S, Howe GA (2012) Role of phytohormones in insect-specific plant reactions. Trends in Plant Science 17: 250-259.

Fahey JW, Zalcmann AT, Talalay P (2001) The chemical diversity and distribution of glucosinolates and isothiocyanates among plants. Phytochemistry 56: 5-51.

Finke DL, Snyder WE (2008) Niche partitioning increases resource exploitation by diverse communities. Science 321: 1488-1490.

Furlong MJ, Wright DJ, Dosdall LM (2013) Diamondback moth ecology and management: problems, progress, and prospects. Annual Review of Entomology 58: 517-541.

Gols R, Harvey JA (2008) Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. Phytochemistry Reviews 8: 187-206.

Gols R, Raaijmakers CE, van Dam NM, Dicke M, Bukovinszky T, Harvey JA (2007) Temporal changes affect plant chemistry and tritrophic interactions. Basic and Applied Ecology 8: 421-433.

Gols R, Wagenaar R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM, Harvey JA (2008) Genetic variation in defense chemistry in wild cabbages affects herbivores and their endoparasitoids. Ecology 89: 1616-1626.

Guerrieri E, Digilio MC (2008) Aphid-plant interactions: a review. Journal of Plant Interactions 3: 223-232.

Hopkins RJ, van Dam NM, Van Loon JJA (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annual Review of Entomology 54: 57-83.

Hughes RD (1963) Population dynamics of the cabbage aphid, *Brevicoryne brassicae* (L.). Journal of Animal Ecology 32: 393-424.

Hunter MD, McNeil JN (1997) Host-plant quality influences diapause and voltinism in a polyphagous insect herbivore. Ecology 78: 977-986.

Johnson MT, Agrawal AA (2005) Plant genotype and environment interact to shape a diverse arthropod community on evening primrose (*Oenothera biennis*). Ecology 86: 874-885.

Kessler A, Halitschke R (2007) Specificity and complexity: the impact of herbivore-induced plant responses on arthropod community structure. Current Opinion in Plant Biology 10: 409-414.

Lepš J, Šmilauer P (2003) Multivariate Analysis of Ecological Data using CANOCO. Cambridge University Press, New York.

Li Y, Dicke M, Harvey J, Gols R (2014) Intra-specific variation in wild *Brassica oleracea* for aphid-induced plant responses and consequences for caterpillar–parasitoid interactions. Oecologia 174: 853-862.

Mithen R, Raybould AF, Giamoustaris A (1995) Divergent selection for secondary metabolites between wild populations of *Brassica oleracea* and its implications for plant-herbivore interactions. Heredity 75: 472-484.

Mooney KA, Agrawal AA (2008) Plant genotype shapes ant-aphid interactions: implications for community structure and indirect plant defense. American Naturalist 171: E195-E205.

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiology 125: 1074-1085.

Mouttet R, Bearez P, Thomas C, Desneux N (2011) Phytophagous arthropods and a pathogen sharing a host plant: evidence for indirect plant-mediated interactions. Plos One 6: doi:10.1371/journal.pone.0018840.

Mouttet R, Kaplan I, Bearez P, Amiens-Desneux E, Desneux N (2013) Spatiotemporal patterns of induced resistance and susceptibility linking diverse plant parasites. Oecologia 173: 1379-1386.

Newton EL, Bullock JM, Hodgson D (2009) Bottom-up effects of glucosinolate variation on aphid colony dynamics in wild cabbage populations. Ecological Entomology 34: 614-623.

Newton EL, Bullock JM, Hodgson DJ (2009) Glucosinolate polymorphism in wild cabbage (*Brassica oleracea*) influences the structure of herbivore communities. Oecologia 160: 63-76.

Ode PJ (2006) Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. Annual Review of Entomology 51: 163-185.

Ohgushi T (2005) Indirect interaction webs: herbivore-induced effects through trait change in plants. Annual Review of Ecology Evolution and Systematics 36: 81-105.

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nature Chemical Biology 5: 308-316.

Poelman EH, Broekgaarden C, Van Loon JJA, 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 EH, Oduor AMO, Broekgaarden C, Hordijk CA, Jansen JJ, Van Loon JJA, Van Dam NM, Vet LEM, Dicke M (2009) Field parasitism rates of caterpillars on *Brassica oleracea* plants are reliably predicted by differential attraction of *Cotesia* parasitoids. Functional Ecology 23: 951-962.

Poelman EH, Van Loon JJA, Van Dam NM, Vet LEM, Dicke M (2010) Herbivore-induced plant responses in *Brassica oleracea* prevail over effects of constitutive resistance and result in enhanced herbivore attack. Ecological Entomology 35: 240-247.

Rodriguez-Saona C, Thaler JS (2005) Herbivore-induced responses and patch heterogeneity affect abundance of arthropods on plants. Ecological Entomology 30: 156-163.

Rodriguez-Saona CR, Musser RO, Vogel H, Hum-Musser SM, Thaler JS (2010) Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. Journal of Chemical Ecology 36: 1043-1057.

Soler R, Badenes-Perez FR, Broekgaarden C, Zheng SJ, David A, Boland W, Dicke M (2012) Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. Functional Ecology 26: 156-166.

Stam JM, Kroes A, Li Y, Gols R, van Loon JJA, Poelman EH, Dicke M (2014) Plant interactions with multiple insect herbivores: from community to genes. Annual Review of Plant Biology 65: 689-713.

ter Braak CJF, Šmilauer P (2012) Canoco Reference Manual and User's Guide, Software for Odination (Version 5.0). Microcomputer Power, Ithaca, NY, USA.

Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science 17: 260-270.

Utsumi S, Ando Y, Miki T (2010) Linkages among trait-mediated indirect effects: a new framework for the indirect interaction web. Population Ecology 52: 485-497.

Van Zandt PA, Agrawal AA (2004) Community-wide impacts of herbivore-induced plant reaponses in milkweed (*Asclepias syriaca*). Ecology 85: 2616-2629.

Van Zandt PA, Agrawal AA (2004) Specificity of induced plant responses to specialist herbivores of the common milkweed *Asclepias syriaca*. Oikos 104: 401-409.

Viswanathan DV, Narwani AJT, Thaler JS (2005) Specificity in induced plant responses shapes patterns of herbivore occurrence on *Solanum dulcamara*. Ecology 86: 886-896.

Walling LL (2008) Avoiding effective defenses: Strategies employed by phloem-feeding insects. Plant Physiology 146: 859-866.

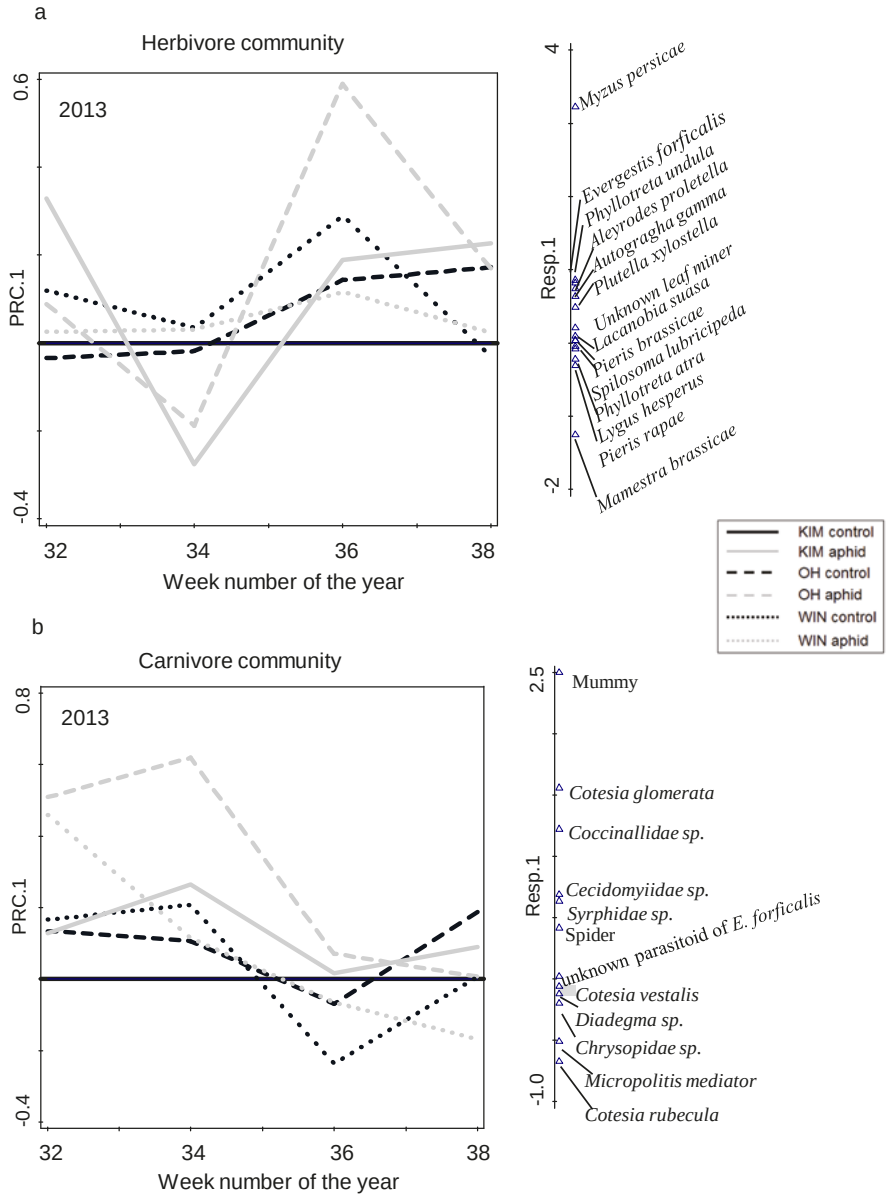
Whitham TG, Bailey JK, Schweitzer JA, Shuster SM, Bangert RK, Leroy CJ, Lonsdorf EV, Allan GJ, DiFazio SP, Potts BM, Fischer DG, Gehring CA, Lindroth RL, Marks JC, Hart SC, Wimp GM, Wooley SC (2006) A framework for community and ecosystem genetics: from genes to ecosystems. Nature Reviews Genetics 7: 510-523.

Whitham TG, Young WP, Martinsen GD, Gehring CA, Schweitzer JA, Shuster SM, Wimp GM, Fischer DG, Bailey JK, Lindroth RL (2003) Community and ecosystem genetics: a consequence of the extended phenotype. Ecology 84: 559-573.

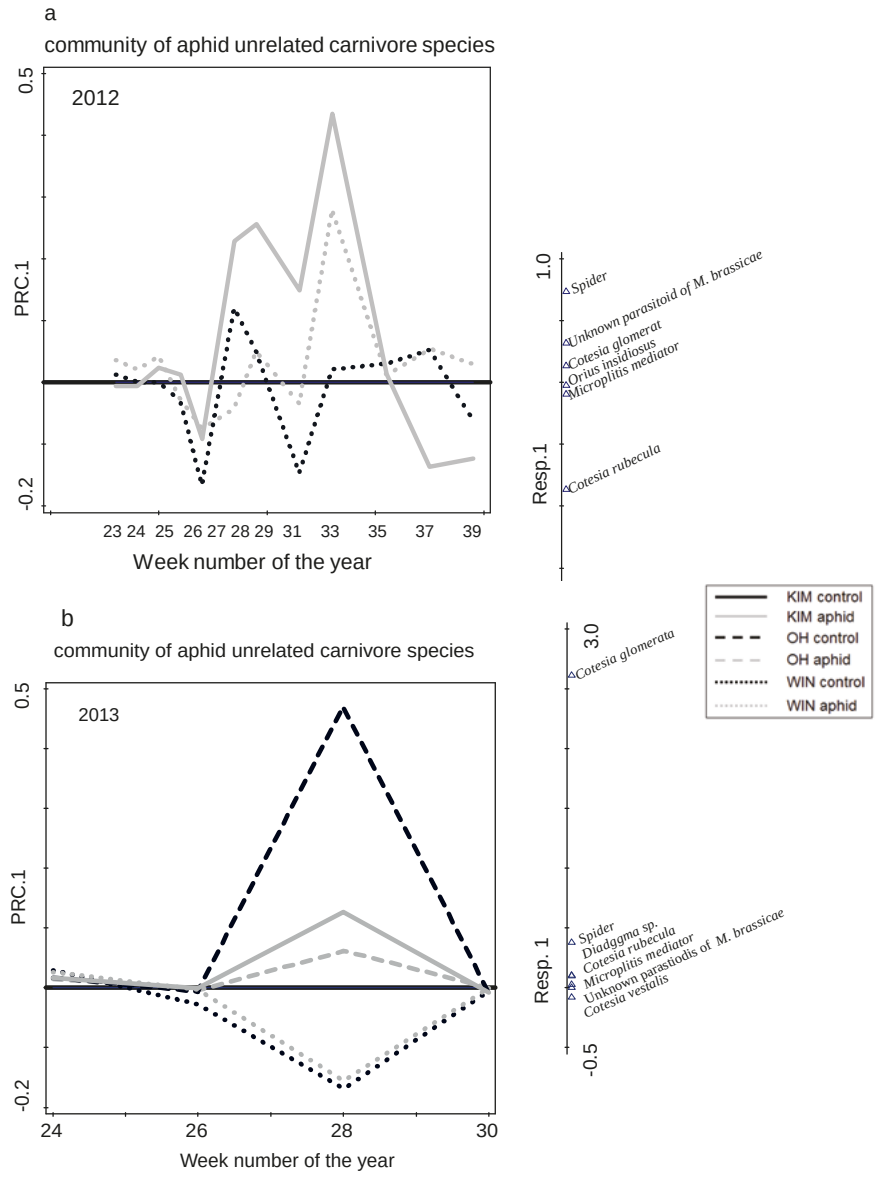
Zarate SI, Kempema LA, Walling LL (2007) Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiology 143: 866-875.

Zeier J (2005) Age-dependent variations of local and systemic defence responses in Arabidopsis leaves towards an avirulent strain of Pseudomonas syringae. Physiological and Molecular Plant Pathology 66: 30-39.

## Appendix




**Appendix 1** Principal response curves (PRC) for the abundance of (a) herbivore species and (b) carnivore species in the second half of the season of 2013 (week 32-38) on plants from wild cabbage (*Brassica oleracea*) populations (KIM, OH and WIN) that were or were not infested with the cabbage aphid (*Brevicoryne brassicae*) early in the season (week 23) (First PRC axis: Monte Carlo permutation test, 499 permutations, explained content: (a) 3.86%, F= 6.7, P= 0.57; (b) 2.01%, F= 3.8, P= 0.16). The KIM control group was arbitrarily set as the baseline against which the other groups were contrasted. Solid lines refer to the KIM, dashed lines to the OH and dotted lines to the WIN population. Grey lines refer to aphid treatment whereas control groups are depicted in black. Species weights on the first principal component are depicted in a score plot on the right side of the figure.




**Appendix 2** Community composition of carnivore species unrelated to aphids, monitored throughout the growing season (week 23-39) in 2012, and in the first half of the season of 2013 (week 24-30) on plants from wild cabbage (*Brassica oleracea*) populations (KIM and WIN in 2012; KIM, OH and WIN in 2013) that were or were not infested with the cabbage aphid (*Brevicoryne brassicae*) early in the season (week 22 in 2012 and week 23 in 2013). Principal Response Curves (PRC) were constructed for the community of carnivore species unrelated to aphids in (a) 2012 and (b) 2013. Solid lines refer to the KIM, dashed lines to the OH and dotted lines to the WIN population. The KIM control group was arbitrarily set as the baseline against which the other groups were contrasted. Grey lines refer to aphid treatment whereas control groups are depicted in black. Species weights on the first principal component are depicted in a score plot on the right side of the figure. (First PRC axis: Monte Carlo permutation test, 499 permutations, explained content: (a) 7.55%, F= 13.7, P= 0.17; (b) 4.26%, F= 15, P= 0.4).

**Appendix 3** Approximate F-test for the fixed effects (plant population, treatment) on the plant morphological traits (plant leaf number, plant height and plant damage score) of three wild *Brassica oleracea* populations with or without infestation of aphids (*Brevicoryne brassicae*) early in the season (week 23 of 2013).

| Leaf number | *n.d.f.* | *d.d.f.* | *F* | *P* |
|---|---|---|---|---|
| Plant population (1) | 2 | 41.4 | 3.56 | **0.037** |
| Treatment (2) | 1 | 41.4 | 0.52 | 0.47 |
| Interaction 1*2 | 2 | 41.4 | 0.2 | 0.82 |
| Plant height | *n.d.f.* | *d.d.f.* | *F* | *P* |
| Plant population (1) | 2 | 42 | 10.16 | **<0.001** |
| Treatment (2) | 1 | 42 | 0.33 | 0.57 |
| Interaction 1*2 | 2 | 42 | 0.06 | 0.94 |
| Plant damage score | *n.d.f.* | *d.d.f.* | *F* | *P* |
| Plant population (1) | 2 | 41.9 | 1.01 | 0.37 |
| Treatment (2) | 1 | 41.9 | 0.1 | 0.75 |
| Interaction 1*2 | 2 | 41.9 | 1.39 | 0.26 |

# Intra-specific variation in wild *Brassica oleracea* for aphid-induced plant responses and consequences for caterpillar-parasitoid interactions

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## Abstract

Herbivore-induced plant responses do not only influence the initiating attackers, but also other herbivores feeding on the same host plant simultaneously or temporally separated. Insects belonging to different feeding guilds are known to induce different responses in the host plant. Changes in the plant's phenotype do not only affect their interactions with herbivores but also with organisms higher up in the food chain. Previous work has shown that feeding by a phloem-feeding aphid facilitates the interaction with a chewing herbivore and its endoparasitoid using a cabbage cultivar. Here we study genetic variation in a plant's response to aphid feeding using plants originating from three wild *Brassica oleracea* populations that are known to differ in constitutive and inducible secondary chemistry. We compared the performance of two different chewing herbivore species, *Plutella xylostella* and *Mamestra brassicae,* and their larval endoparasitoids *Diadegma semiclausum* and *Microplitis mediator,* respectively, on plants that had been infested with aphids (*Brevicoryne brassicae*) for one week already. Remarkably, early infestation with *B. brassicae* enhanced the performance of the specialist *P. xylostella* and its parasitoid *D. semiclausum*, but did not affect that of the generalist *M. brassicae*, nor its parasitoid *M. mediator*. Performance of the two herbivore-parasitoid interactions also varied among the cabbage populations and the effect of aphid infestation marginally differed among the three populations. Thus, the effect of aphid infestation on the performance of subsequent attackers is species specific, which may have concomitant consequences for the assembly of insect communities that are naturally associated with these plants.

**Keywords:** Cabbage, *Diadegma semiclausum,* herbivory, leaf chewers, *Mamestra brassicae*, *Microplitis mediator,* phloem feeders, plant induction, *Plutella xylostella*.

## Introduction

In nature, plants are often attacked by multiple herbivores either simultaneously or at different times (Ohgushi 2005; Dicke et al. 2009; Ponzio et al. 2013). Herbivory may lead to the enhanced production of secondary metabolites and defence proteins, as well as changes in morphological traits, such as spines, trichomes and wax layers (Awmack and Leather 2002; Schoonhoven et al. 2005). These inducible plant defences have been shown to mediate interactions among herbivores that feed on a common host plant but which are temporally separated (Ohgushi et al. 2007; Poelman et al. 2008; Dicke et al. 2009). Damage inflicted on a plant by an early colonizing herbivore has been shown to influence the development and behaviour not only of subsequent herbivores but also that of their natural enemies (Rodriguez-Saona et al. 2005; Gols and Harvey 2009; Poelman et al. 2010; Poelman et al. 2011a; Soler et al. 2012).

Plant-mediated effects on the performance of herbivores that feed on the plant successively are often, but not always (Mayer et al. 2002), negative when the herbivore species belong to the same feeding guild (Denno et al. 1995; Inbar et al. 1999; Agrawal 2000b; Lynch et al. 2006; Kaplan and Denno 2007). However, facilitation of subsequently colonising herbivores has also been observed, for example when the herbivores belong to different feeding guilds (Soler et al. 2012). In the latter situation, the predominant mechanism is that the first attacking herbivore attenuates the induced plant defence against a subsequent herbivore when the second species belongs to a different feeding guild (Kessler and Baldwin 2004; Zarate et al. 2007; Dicke et al. 2009; Rodriguez-Saona et al. 2010).



Attenuated plant defence may benefit not only insect herbivores but it may also be advantageous to the performance of their natural enemies consuming these herbivores (Soler et al. 2012). Conversely, herbivore feeding may enhance plant defences against a second species and its natural enemies when the two herbivores belong to the same feeding guild. For example, two larval endoparasitoids of *P. xylostella* hosts were smaller and grew at a lower rate on *Brassica oleracea* plants previously damaged by *P. rapae* than on control plants (Bukovinszky et al. 2012b).

Many studies exploring plant defences in response to single or multiple herbivores have been performed on either *Arabidopsis thaliana* (Pieterse and Dicke 2007; Koornneef et al. 2008; Zhang et al. 2013) or cultivated crop plants (Rodriguez-Saona et al. 2010Dafoe et al. 2011; Soler et al. 2012), whereas, few studies have explored these processes in wild species (e.g. Agrawal 2000a; Kessler and Baldwin 2004; Voelckel and Baldwin 2004). *Arabidopsis thaliana* is a good model plant for studying the mechanisms of plant defences at the molecular level, but may be less suitable to study ecological interactions with insects and their natural enemies with which it rarely interacts in nature (Harvey et al. 2007; Pieterse and Dicke 2007). Considering that phenotypic traits in crop plants are the result of artificial selection, the use of cultivars in understanding ecological interactions in an evolutionary perspective is open to conjecture (Gols et al. 2008; Gols and Harvey 2009; Hopkins et al. 2009).

In this study we investigated herbivore-induced plant responses in wild cabbage (*Brassica oleracea*) plants originating from populations, which grow naturally along the rocky coastlines of Dorset, Great Britain. These wild populations of *B. oleracea* differ in constitutive and inducible levels of glucosinolates, secondary metabolites characteristic for Brassicaceae that play an important role in defence against a range of attackers including insects (Moyes et al. 2000; Gols et al. 2008; Newton et al. 2009a; Newton et al. 2009b; Harvey et al. 2011). This natural variation in secondary chemistry and possibly also in other defence traits has been demonstrated to affect the performance (Gols et al. 2008; Harvey et al. 2011; Abdalsamee and Müller 2012) and the community structure (Moyes and Raybould 2001; Newton et al. 2009b) of insects associated with these wild cabbage populations. For example, both among and within the wild cabbage populations, the infestation rate with the specialist herbivores, the aphid *Brevicoryne brassicae* and caterpillars of the large cabbage white *Pieris brassicae,* negatively correlated with the presence of the glucosinolate sinigrin, whereas no correlation was found between glucosinolate chemistry and infestation with the cabbage moth *Mamestra brassicae* (Newton et al., 2009b). In the present study we investigated intraspecific variation in herbivore-induced plant responses when a plant is challenged by two herbivores, each belonging to a different feeding guild.

The objective of this study was to investigate variation in the response to herbivory among three selected wild populations that are known to vary in their defence chemistry (Gols et al. 2008; Harvey et al. 2011). More specifically, we investigated 1) whether initial infestation with the cabbage aphid *Brevicoryne brassicae* L. (Hemiptera: Aphididae) facilitates the performance of caterpillars that arrive later, and 2) whether this facilitation cascades up to the third trophic level. We compared fitness correlates (biomass and development time) of two species of leaf-chewing herbivores and two associated larval endoparasitoids. We selected the diamondback moth *Plutella xylostella* L. (Lepidoptera: Plutellidae), which is a crucifer specialist, together with one of its larval endoparasitoids, *Diadegma semiclausum* Helèn (Hymenoptera: Ichneumonidae). In addition, we quantified the performance of the cabbage moth *Mamestra brassicae* L. (Lepidoptera: Noctuidae), which has a much broader diet, and its larval endoparasitoid *Microplitis mediator* Haliday (Hymenoptera: Braconidae). All insects used in this study are associated with brassicaceous plants in nature.



## Material and Methods

### Plants

Seeds of the three wild cabbage populations were collected in Dorset at sites known as 'Old Harry' (50°38'N, 1°55'W), 'Kimmeridge' (50°36'N, 2°07'W), and 'Winspit' (50°35'N, 2°02'W). Hereafter, the populations were abbreviated as OH (Old Harry), WIN (Winspit) and KIM (Kimmeridge). Wild cabbage seeds were sown five weeks before the start of the

experiment. One week after seed germination, seedlings of the three populations were transferred to 2-L pots (one seedling per pot) filled with potting soil ('Lentse potgrond' no. 4; Lent, The Netherlands) and grown in a greenhouse (22 ± 3 °C, 50-70% relative humidity, L16:D8). Supplementary illumination was supplied by high pressure mercury lamps when the natural light intensity dropped below the threshold of 500 µmol photons /m2 /s during the 16-h photoperiod. Plants were watered daily.

### Insects

All insects used in the experiments were reared on Brussels sprouts plants (*B. oleracea*, var. *gemmifera*, cv. Cyrus) in climate rooms (22 ± 1 °C, 50-70% R.H. and a L16:D8) or in greenhouses (22 ± 3 °C, 50-70% R.H. and L16:D8). All insects were originally collected from agricultural cabbage fields near Wageningen University, The Netherlands. The two parasitoid species were reared on plants heavily infested with host caterpillars. Approximately one hundred newly emerged parasitoids were collected in a 1:1 male-to-female sex ratio, 3-4 days before the start of the experiment to allow mating. These parasitoids were maintained at room temperature and supplied with honey as an energy source.

### Experimental protocol

The bioassays with *P. xylostella* and *D. semiclausum* were performed separately from those with *M. brassicae* and *M. mediator*, each with 40 plants per population allocated to one of two plant treatments (with or without aphids). The performance of each insect was studied using 10 plants per population and plant treatment. When the plants were 4 weeks old, half of the plants of each population were inoculated with five adult *B. brassicae* aphids, which were randomly selected from the rearing. The other half of the plants remained aphid-free (= control), but were otherwise treated similarly as the aphid-infested plants. Aphids were placed on the youngest fully expanded leaf of each plant and were allowed to move, feed and reproduce freely for 7 days before the experimental insects (*P. xylostella* or *M. brassicae* caterpillars) were introduced (see below). The aphids were counted 7 days after inoculation, just before infesting plants with healthy or parasitized *P. xylostella* larvae. Aphids remained on the plants throughout the experiment. To prevent cross contamination or escaping of insects, each plant was enclosed within a nylon sleeve net (size: 48×60 cm, nylon netting 104×94 mesh, Bugdorm, Taiwan), which was supported by four wooden sticks and was tightly attached around the rim of the pots.

To obtain oviposition substrates for *P. xylostella* eggs, folded pieces of Parafilm (20×5 cm) were treated with cabbage juice. Approximately 65 g of cabbage leaves was thoroughly blended with 500 ml tap water using a food processor. The Parafilm sheets were dipped into this juice, air-dried, and were then used to seal and cover the inner wall of a plastic cylinder (length 25cm x diameter 4.5 cm) containing approximately 120 *P. xylostella* adults

in a 1:1 male-to-female sex ratio. Females were allowed to oviposit on the Parafilm sheets overnight in a climate cabinet (22 ± 1°C, L16:D8). The egg sheets were cut into strips carrying approximately 20 eggs, which were used in the experiment (see below). *Mamestra brassicae* females do not need plants as a substrate for oviposition, and readily lay eggs on paper. Sheets of paper with *M. brassicae* eggs were obtained from the general insect rearing, and incubated in a climate cabinet (25± 1°C, L16:D8) for 4 days until the eggs hatched.

To obtain second-instar (L2) caterpillars of both herbivore species that had been feeding on plants of each of the three *B. oleracea* populations, either treated with aphids or not, Parafilm strips with *P. xylostella* eggs (70-80 per plant) or paper sheets with *M. brassicae* neonates (50-60 per plant) were distributed over three plants of each of the six population-treatment combinations. The groups of plants were placed separately in six cages in a greenhouse (22 ± 3 °C, 50-70% R.H. and L16:D8). When the caterpillars had reached the L2 stage (six to seven days after oviposition), a cohort of 100 healthy *P. xylostella* larvae or 40 healthy *M. brassicae* were collected randomly from each of the population-treatment combinations, and transferred to 10 new plants (10 *P. xylostella* or 4 *M. brassicae* larvae per plant) of their respective population and treatment. The caterpillars were initially introduced onto the younger leaf nearest to the one that had been inoculated with aphids, but were then allowed to move and feed freely on the plants. Caterpillars in the control group (plants without aphids) were introduced onto a leaf of a similar age as in the aphid-treated plants.

For both herbivore species, a second cohort of 100 larvae (L2) was collected from each population-treatment combination and parasitized by its respective parasitoid (*D. semiclausum* for *P. xylostella* and *M. mediator* for *M. brassicae*). For parasitism, a single host caterpillar was presented to a female wasp in a glass vial. The host was considered parasitized when the female inserted and removed her ovipositor from the larva, which generally took one to a few seconds. Each female wasp was allowed to parasitize a maximum of ten hosts. After parasitism, the caterpillars were transferred to new plants (10 caterpillars per plant) of the same population and treatment as they had been feeding on previously. The caterpillars were initially introduced onto the younger leaf nearest to the one that had been inoculated with aphids or a leaf of similar age of the control plants, but were then allowed to move and feed freely on the plants until pupation.

Pupae (healthy *P. xylostella*) and cocoons (*D. semiclausum*) were collected from the plants and placed in labelled glass vials. When adult emergence was approaching, vials were monitored every 2 hours, and the time of emergence (in days) as well as the insect's sex was recorded. Development time was determined as the number of days between *P. xylostella* egg deposition or *D. semiclausum* parasitism and adult eclosion. Newly emerged adults were killed by freezing at -20 °C, subsequently dried for 2 days in an oven at 80°C and

weighed individually on a microbalance (Sartorius CP2P; Germany). *Mamestra brassicae* pupates in the soil. Therefore, healthy larvae in the late final (L5) stage were transferred to plastic containers (15×12×6cm) filled with a 1-cm layer of potting soil mixed with vermiculite (1:1) and some fresh leaf material from the plant on which they had been feeding. Once the caterpillars had burrowed into the vermiculite-soil mixture, boxes were monitored daily and pupae were collected and weighed on an analytical balance (accuracy ± 0.1 mg, Newclassic MF, Mettler Toledo, Switzerland). For each pupa, the time between hatching and pupation was also recorded. Cocoons of *M. mediator* were collected and monitored as described for *D. semiclausum*.

### Statistics

The number of aphids 7 days after inoculation was compared on the three plant populations using one-way ANOVA. Survival to pupation of the herbivores and the parasitoids that were initially introduced onto the plant was compared using a generalized linear model with a binomial distribution for errors and a logit link function (=logistic regression). Plant population, aphid induction and their interaction were entered as fixed factors in the model. The fitness correlates biomass and development time of the two herbivores and their parasitoids were analysed using a mixed model with fixed and random variables. In all analyses each plant served as an experimental unit. Plant population (KIM, OH, WIN) and plant treatment (aphids and control) and their interaction were entered as fixed effects, whereas plant individual nested within plant population-treatment was entered as a random effect. In the analysis of *P. xylostella*, *D. semiclausum*, and *M. mediator* data, we also included sex as a third fixed factor. Model fit was done by employing restricted maximum likelihood (REML) in SAS 9.2 and statistical tests for fixed effects were done using approximated *F*-tests. When interaction terms were not significant, they were sequentially removed from the statistical model. Tukey-Kramer multiple comparisons between means were conducted when any of the main factors was significant. Development times of *P. xylostella* and *D. semiclausum* were log-transformed to meet assumptions of normality and equal variance. Analyses were performed using the statistical software SAS version 9.2



## Results

### Performance of *Plutella xylostella* and its parasitoid *Diadegma semiclausum*

Aphid numbers, counted 7 days after inoculation, did not vary among the cabbage populations ($F_{2,57}$ = 0.91, $P$= 0.41; mean ± SE, 29.92 ± 1.39). Survival to pupation of *P. xylostella* varied between 81 and 89% and was not affected by plant population ($\chi^2_2$ = 1.43, $P$= 0.24) or aphid treatment ($\chi^2_1$ = 1.43, $P$= 0.24). Plant treatment had a significant effect

on the biomass of adult *P. xylostella* (Table 1). Overall, *P. xylostella* adults were 8% heavier on aphid-infested than on control plants (Fig 1a). Regardless of plant population and treatment, females were heavier than males (Table 1). Plant population had a strong effect on egg-to-adult development time of *P. xylostella* (Table 1). Moreover, the interaction between population and treatment was also significant. Aphid induction had no effect on development time when the moths were reared on KIM and WIN plants, whereas moths developed significantly slower on control than on aphid-infested OH plants (Fig 1c).

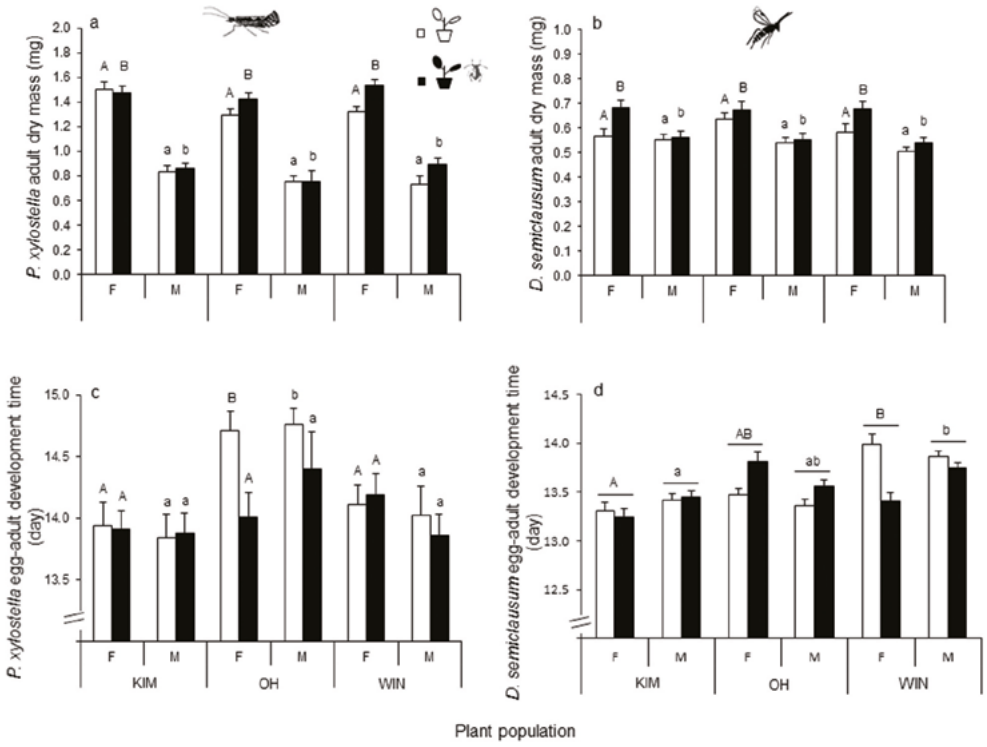


**Figure 1.** Performance of the insect herbivore *Plutella xylostella* and its larval endoparasitoid *Diadegma semiclausum* on three wild cabbage populations ("KIM", "WIN" or "OH") either infested with aphids (*Brevicoryne brassicae*) for seven days previously or left aphid free. Mean dry mass (± SE) of (a) *P. xylostella*, and (b) *D. semiclausum* adults; mean egg-to-adult development time (± SE) of (c) *P. xylostella,* and (d) *D. semiclausum;* male (M) and female (F) data when the insects were reared on uninfested (white bars) or aphid infested (black bars) *Brassica oleracea* plants originating from wild populations in Dorset, England. The multiple comparisons between means are indicated by upper case letters for female insects and lower case letters for male insects; different letters indicate significant differences between means. Per plant population-treatment combination, the number of *P. xylostella* adults varied between 6 and 26 and for *D. semiclausum* adults between 9 and 38.

Of the initially parasitized caterpillars between 52 and 73% produced *D. semiclausum* cocoons. Successful pupation was not affected by plant population ($\chi^2_2 = 0.59$, $P = 0.56$) or aphid treatment ($\chi^2_1 = 0.42$, $P = 0.52$). As for the host *P. xylostella*, the treatment with aphids also affected the adult biomass of *D. semiclausum* (Table 1, Fig 1b); the biomass of wasps

was 9% higher when they developed in hosts on aphid-infested plants. Overall, the females were heavier than the males (Table 1, Fig 1b). Plant population did affect the development time but not adult biomass of *D. semiclausum* (Table 1, Fig 1b, d). Parasitoids developed significantly faster on KIM than on WIN plants, whereas development time on the OH plants was intermediate and not significantly different from the development times on the other two populations (Fig 1d).

### Performance of *Mamestra brassicae* and its parasitoid *Microplitis mediator*

Survival to pupation of *M. brassicae* varied between 40 and 68% and was not affected by plant population ($\chi^2_2 = 0.85$, $P = 0.43$) or aphid treatment ($\chi^2_1 = 0.81$, $P = 0.37$). Biomass of *M. brassicae* pupae varied only with the population on which the insects had been reared and

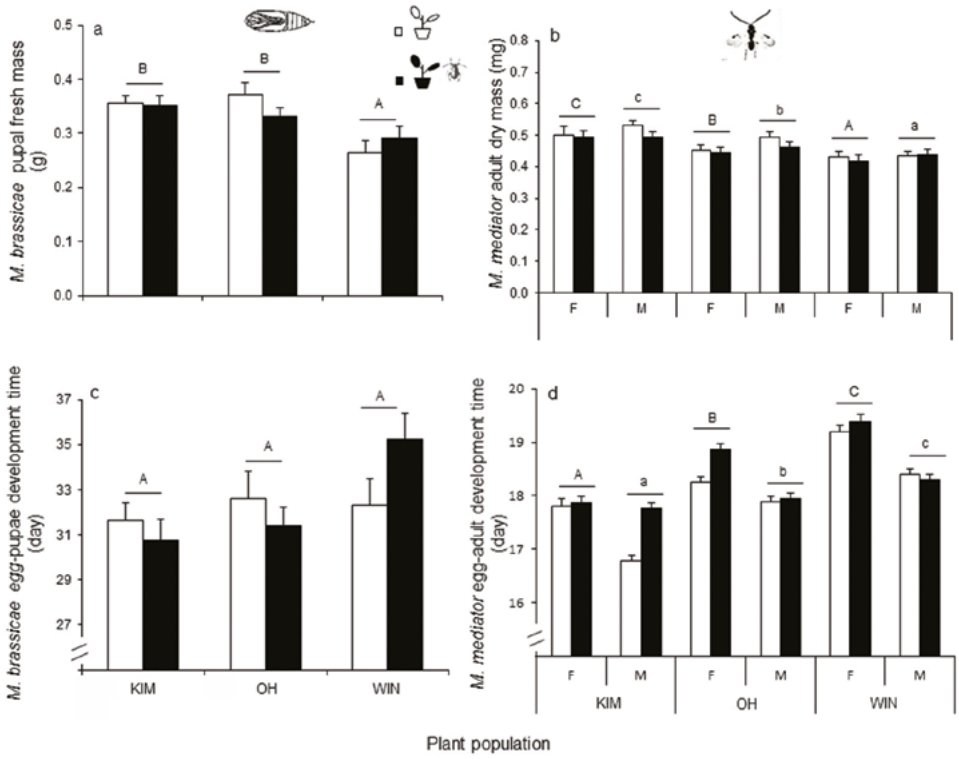


**Figure 2.** Performance of the insect herbivore *Mamestra brassicae* and its larval endoparasitoid *Microplitis mediator* on three wild cabbage populations ("KIM", "WIN" or "OH") either infested with aphids (*Brevicoryne brassicae*) for seven days previously or left aphid free. Mean (± SE) fresh mass of *M. brassicae* pupae (a), mean dry mass of *M. mediator adults* (b); mean larva-to-pupa development time of *M. brassicae* (c), mean egg-to-adult development time of *M. mediator* (d); male (M) and female (F) data when the insects were reared on uninfested (white bars) or aphid infested (black bars) *Brassica oleracea* plants originating from wild populations in Dorset, England. The multiple comparisons between means are indicated by upper case letters for *D. semiclausum* females and lower case letters for the males; different letters indicate significant differences between means. Per plant population-treatment combination, the number of *M. brassicae* pupae varied between 8 and 21 and for *M. mediator* adults between 32 to 69.

not with plant treatment (Table 1, Fig 2a). Pupal mass was comparable on KIM and OH and approximately 20 % lower on WIN plants. Development time of *M. brassicae* pupae was highly variable and was not affected by plant population ($P$ = 0.06) or aphid treatment (Fig 2c, Table 1).

**Table 1.** Approximate *F*-test for the fixed effects (population, plant treatment, and insect sex) on the fitness correlates biomass and development time of two insect herbivore species and their larval endoparasitoids, *Plutella xylostella* - *Diadegma semiclausum*, and *Mamestra brassicae* - *Microplitis mediator*, respectively. The non-significant interactions terms were removed from the statistical model. Insect had developed on wild *Brassica oleracea* plants originating from one of three populations and the plants had been infested with aphids (*Brevicoryne brassicae*) or remained aphid free. Bold typeface indicates significant terms (α =0.05).

| Insect species | Variable | Factor | N.d.f. | D.d.f. | *F*-statistic | *P*-value |
|---|---|---|---|---|---|---|
| ***P. xylostella*** | Biomass | Plant population | 2 | 52.4 | 2.41 | 0.10 |
| | | Treatment | 1 | 55.8 | 6.51 | **0.014** |
| | | Sex | 1 | 169 | 476 | **<0.001** |
| | Development time | Plant population (1) | 2 | 53.5 | 8.71 | **<0.001** |
| | | Treatment (2) | 1 | 54.8 | 3.96 | 0.052 |
| | | Sex | 1 | 172 | 0.18 | 0.67 |
| | | (1)*(2) | 2 | 54.7 | 3.23 | **0.047** |
| ***D. semiclausum*** | Biomass | Plant population | 2 | 53 | 1.19 | 0.31 |
| | | Treatment | 1 | 52.5 | 6.65 | **0.013** |
| | | Sex | 1 | 238 | 47.6 | **<0.001** |
| | Development time | Plant population | 2 | 54.4 | 5.45 | **0.007** |
| | | Treatment | 1 | 53.8 | 0.03 | 0.87 |
| | | Sex | 1 | 239 | 0.21 | 0.65 |
| ***M. brassicae*** | Biomass | Plant population | 2 | 32 | 8.29 | **0.001** |
| | | Treatment | 1 | 31.1 | 0.20 | 0.66 |
| | Development time | Plant population | 2 | 35.1 | 2.96 | 0.065 |
| | | Treatment | 1 | 34.1 | 0.01 | 0.96 |
| ***M. mediator*** | Biomass | Plant population | 2 | 54.1 | 14.2 | **<0.001** |
| | | Treatment | 1 | 54.3 | 2.09 | 0.15 |
| | | Sex | 1 | 324 | 4.33 | **0.038** |
| | Development time | Plant population | 2 | 53.2 | 17.3 | **<0.001** |
| | | Treatment | 1 | 53.4 | 3.07 | 0.085 |
| | | Sex | 1 | 319 | 30.5 | **<0.001** |

Survival to pupation of *M. mediator* was homogenous across the plant populations ($\chi^2_2$ = 2.27, $P$= 0.10) and treatments ($\chi^2_1$ = 1.70, $P$= 0.19), and varied between 54 and 68%. Plant population and aphid treatment affected the adult biomass of *M. mediator* wasps similarly to what was observed for the healthy hosts, i.e. the effect of plant population was significant, whereas the effect of aphid treatment was not (Table 1). The heaviest *M. mediator* wasps emerged from hosts that were reared on KIM plants; the mass of wasps was intermediate on OH plants and lowest on WIN plants (Fig 2b). Females of this species are lighter than the males (Table 1, Fig 2b). Host-plant population also had a significant effect on egg-to-adult development time of *M. mediator* (Fig 2d). Development time of *M. mediator* was shortest on KIM, longer on OH and longest on WIN plants (Fig 2d) and the

males developed faster than the females (Table 1). Treatment with aphids did not affect egg-to-adult development time of *M. mediator* (Table 1).

## Discussion

Plant quality can change in response to insect herbivory (Karban and Baldwin 1997; Agrawal 1999). Results of the present study demonstrate that the effect of aphid induction on herbivore and parasitoid performance differed among the plants originating from the three wild cabbage populations. More importantly, the effect of aphid-induced changes in the plants on subsequent herbivore-parasitoid interactions was species specific. Aphid-induced differences in plant quality positively affected the development of the specialist herbivore *P. xylostella* and its parasitoid *D. semiclausum*, but did not significantly influence the development of the generalist herbivore *M. brassicae* and its parasitoid *M. mediator.*

Plants may respond differently depending on the feeding patterns of the attacking herbivore (Ohgushi 2005; Schoonhoven et al. 2005), or on the salivary components that come into contact with plant tissues during feeding (Diezel et al. 2009; Bonaventure et al. 2011). These differences are translated at the molecular level in the activation of different signalling pathways. Chewing insects primarily activate jasmonic acid or JA-dependent defences, whereas sucking herbivores activate salicylic acid or SA-dependent defences (Zhang et al. 2009; Ponzio et al. 2013). Moreover, antagonistic cross-talk of plant signalling pathways may occur when these two types of herbivores attack the same plant individual (Heil and Ton 2008; Pieterse et al. 2012). For example, Soler et al. (2012) reported that cultivated Brussels sprouts (*B. oleracea*) plants infested with *B. brassicae* enhanced the performance of the caterpillar *P. brassicae* and its parasitoid *C. glomerata*, due to the suppression of JA-dependent defences in response to aphid feeding. Similarly, the larval growth rate of *Spodoptera exigua* was higher on tomato plants infested with aphids (*Macrosiphum euphorbiae*) than on undamaged control plants (Stout et al. 1997; Rodriguez-Saona et al. 2005).

Aphid infestation has been demonstrated to enhance the nutritional quality of host plants through the alteration of plant secondary metabolism, nitrogen allocation and assimilation. For example, aphid feeding can increase the amino acid concentration of the phloem sap (Sandstrom et al. 2000; Thompson and Goggin 2006), which in turn may improve the nutritional quality of the host plant not only for the aphids but also for chewing herbivores. The plant's response to herbivory is not restricted to the wounded tissues, but systemically spreads to distant, unwounded parts of the plant (Heil and Ton 2008). This systemic response is the result of the translocation of signals associated with plant defence. For example, systemic acquired resistance (SAR), usually associated with pathogen infections, involves accumulation of SA at the site of pathogen infection, followed by SA upregulation

in younger leaves, resulting in SAR in younger leaves (Zeier 2005; Heil and Ton 2008). Moreover, systemic responses also include the transport of defensive metabolites from the wounded tissue through the phloem and xylem to other parts of the plant in the direction to the younger shoots (Stratmann 2003; Heil and Ton 2008). In response to aphid feeding, the biosynthesis of metabolites (glucosinolates, terpenoids) and proteins (protease inhibitors) increases, which in turn may compromise the performance of insect herbivores feeding on these tissues (Thompson and Goggin 2006; Kusnierczyk et al. 2008). Thus, the aphid-induced changes in both nutrients and defence compounds are likely to be relatively higher in younger than in older leaves.

We recorded differential performance effects for two herbivore species belonging to the same feeding guild, but with different dietary specialization. The specialist *P. xylostella* was observed to mainly feed on young leaves throughout its immature development, which are highly nutritious and well-defended, whereas the generalist *M. brassicae* was restrictively feeding on older leaves that contain lower levels of deleterious secondary metabolites and nutrients (Lambdon et al. 2003; Gols et al. 2007). Specialist herbivores are predicted to be better adapted to plant defences than generalist herbivores (Ali and Agrawal 2012). The specialist *P. xylostella* is capable of detoxifying glucosinolates via an endogenous sulfatase (Ratzka et al. 2002), whereas the generalist *M. brassicae* metabolise glucosinolate-derived products by glutathione transferases and mixed function oxidases (Schramm et al. 2012). However, specialists detoxify these adverse metabolites more efficiently than generalists (Winde and Wittstock 2011). Aphid infestation may have had little effect on the performance of the generalist *M. brassicae* because they selectively feed on older leaves, in which the effect of aphid-induction is predicted to be limited as explained in the previous section. In contrast, aphid infestation had significant positive effects on the performance of the specialist *P. xylostella*. This herbivore can cope with increased levels of secondary metabolites and at the same time benefit from the higher nutrient levels in the young leaves. Thus, in this 'battle-field' of co-evolution of plants and herbivores, both parties show adaptation and plasticity. Our results suggest that it is important to consider dietary preference of herbivores as a 'counter-defence' in the picture of plant-insect interactions and not just consider dietary breadth.

Herbivore-induced plant changes may not only affect the inducing herbivore or subsequently arriving herbivores (Agrawal 2000b; Poelman et al. 2008), they have also been shown to influence the performance of parasitoids (Gols and Harvey 2009; Poelman et al. 2011a). The performance of parasitoids is directly influenced by quantitative changes in host quality and is often mediated through the diet of the host (Gols et al. 2009; Harvey et al. 2011). As the development of the parasitoid offspring is strongly physiologically synchronised with that of the host, the performance of the host and the parasitoid are often positively correlated (Harvey 2005). An exception to this rule is a study by Harvey and Gols (2011) using the same wild cabbage populations where the performance of *M. mediator* was much

stronger affected by previous feeding by *Pieris rapae* caterpillars than the performance of healthy unparasitized *M. brassicae* hosts. The results of the present study and those reported by Harvey and Gols (2011) demonstrate that the outcome of phenotypic plant changes in response to herbivory depends on the inducing agent and the herbivore-parasitoid interaction. This may have consequences for the assembly of associated insect communities. However, parasitized and unparasitized caterpillars were introduced onto the host plants and confined to complete their life cycle on that plant. In future studies investigating bottom-up and top-down control of insect herbivores, it is necessary to also examine the oviposition preference of both the herbivores and their parasitoids (Poelman et al. 2011b). This will further contribute to our understanding explaining herbivore and parasitoid dynamics in a community framework in a system that has evolved under natural selection.

Aphid infestation did not appear to affect the three populations similarly. Variable plant responses to aphid infestation may also be the result of differences in aphid performance caused by morphological and chemical differences. Aphid numbers did not differ among the three cabbage populations one week after introduction. Therefore, the observed differential performance of the *P. xylostella* and *D. semiclausum* on aphid-infested cabbage populations is likely to be mediated by intra-specific plant variation in response to aphid feeding, and not by differences in the performance of the aphids themselves.



Inducible plant responses to herbivory are insect-density dependent (Zehnder and Hunter 2007), and this may have consequences for the interactions between multiple herbivores attacking a common host plant. Denno et al. (1995) and Rodriguez-Saona et al. (2005) argued that the transient benefits for caterpillars feeding on aphid-infested plants would be compromised by competition with a growing aphid population. Thus, it would be interesting to investigate aphid-induced effects on the performance of chewing herbivores at different aphid densities. Moreover, some studies have shown that plants infested by aphids attract parasitoids of caterpillars, because these wasps can feed on aphid honeydew (Faria et al. 2008; Kugimiya et al. 2010). However, volatile-mediated foraging by parasitoids may be negatively affected when a plant is not only damaged by host but also by non-host herbivores, thus, reducing foraging efficiency of the wasps (de Rijk et al. 2013; Zhang et al. 2009; Bukovinszky et al. 2012a).

In natural communities, the amount of herbivore damage and the number of herbivore species attacking a plant can vary dramatically from one plant to another. In turn, the number of natural enemies – predators, parasitoids and hyperparasitoids – associated with these herbivores can vary by several factors. Even within patches where the same plant species grows in aggregations, these parameters may differ quite markedly in neighboring plants. Individual brassicaceous plants in nature are known to harbor several species of chewing and phloem feeding herbivores. On single wild cabbage WIN plants in the UK, for example, we have found whiteflies, aphids and several species of lepidopteran herbivores

(R. Gols and J.A. Harvey, personal observations). Therefore, there may be considerable heterogeneity and variation in trophic interactions that are manifested even at relatively small scales. Adding to this complexity is the fact that each herbivore species may induce different plant responses and these may cascade through the entire trophic chain and lead to broader community-wide effects. In this context, studies exploring interactions involving multiple herbivores and their natural enemies will greatly add to our understanding of plant responses and their consequences for trophic interactions and ecological communities in a broader ecophysiological framework.

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## References


Abdalsamee MK, Müller C (2012) Effects of indole glucosinolates on performance and sequestration by the sawfly *Athalia rosae* and consequences of feeding on the plant defense system. Journal of Chemical Ecology 38: 1366-1375.

Agrawal AA (1999) Induced plant defense: evolution of induction and adaptive phenotypic plasticity. In Agrawal AA, Tuzun S, Bent E (Eds.) *Induced Plant Defenses Against Pathogens and Herbivores. Biochemstry, Ecology and Agriculture* (pp. 251-268). St. Paul, Minosota: APS Press.

Agrawal AA (2000a) Specificity of induced resistance in wild radish: causes and consequences for two specialist and two generalist caterpillars. Oikos 89: 493-500.

Agrawal AA (2000b) Benefits and costs of induced plant defense for *Lepidium virginicum* (Brassicaceae). Ecology 81: 1804-1813.

Ali JG, Agrawal AA (2012) Specialist versus generalist insect herbivores and plant defense. Trends in Plant Science 17: 293-302.

Awmack CS, Leather SR (2002) Host plant quality and fecundity in herbivorous insects. Annual Review of Entomology 47: 817-844.

Bonaventure G, VanDoorn A, Baldwin IT (2011) Herbivore-associated elicitors: FAC signaling and metabolism. Trends in Plant Science 16: 294-299.

Bukovinszky T, Poelman EH, Kamp A, Hemerik L, Prekatsakis G, Dicke M (2012a) Plants under multiple herbivory: consequences for parasitoid search behaviour and foraging efficiency. Animal Behaviour 83: 501-509.

Bukovinszky T, Gols R, Smid HM, Bukovinszkine Kiss G, Dicke M, Harvey JA (2012b) Consequences of constitutive and induced variation in the host's food plant quality for parasitoid larval development. Journal of Insect Physiology 58: 367-375.

Dafoe NJ, Huffaker A, Vaughan MM, Duehl AJ, Teal PE, Schmelz EA (2011) Rapidly induced chemical defenses in maize stems and their effects on short-term growth of *Ostrinia nubilalis*. Journal of Chemical Ecology 37: 984-991.

De Rijk M, Dicke M, Poelman EH (2013) Foraging behaviour by parasitoids in multiherbivore communities. Animal Behaviour 85: 1517-1528.

Denno RF, McClure MS, Ott JR (1995) Interspecific interactions in phytophagous insects: competition reexamined and resurrected. Annual Review of Entomology 40: 297-331.

Dicke M, Van Loon JJA, Soler R (2009) Chemical complexity of volatiles from plants induced by multiple attack. Nature Chemical Biology 5: 317-324.

Diezel C, von Dahl CC, Gaquerel E, Baldwin IT (2009) Different Lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. Plant Physiology 150: 1576-1586.

Faria CA, Wäckers FL, Turlings TCJ (2008) The nutritional value of aphid honeydew for non-aphid parasitoids. Basic and Applied Ecology 9: 286-297.

Gols R, Harvey JA (2009) Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. Phytochemistry Reviews 8: 187-206.

Gols R, van Dam NM, Raaijmakers CE, Dicke M, Harvey JA (2009) Are population differences in plant quality reflected in the preference and performance of two endoparasitoid wasps? Oikos 118: 733-743.

Gols R, Raaijmakers CE, van Dam NM, Dicke M, Bukovinszky T, Harvey JA (2007) Temporal changes affect plant chemistry and tritrophic interactions. Basic and Applied Ecology 8: 421-433.

Gols R, Wagenaar R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM, Harvey JA (2008) Genetic variation in defense chemistry in wild cabbages affects herbivores and their endoparasitoids. Ecology 89: 1616-1626.

Harvey JA (2005) Factors affecting the evolution of development strategies in parasitoid wasps: the importance of functional constraints and incorporating complexity. Entomologia Experimentalis et Applicata 117: 1-13.

Harvey JA, Gols R (2011) Population-related variation in plant defense more strongly affects survival of an herbivore than its solitary parasitoid wasp. Journal of Chemical Ecology 37: 1081-1090.

Harvey JA, Witjes LMA, Benkirane M, Duyts H, Wagenaar R (2007) Nutritional suitability and ecological relevance of *Arabidopsis thaliana* and *Brassica oleracea* as foodplants for the cabbage butterfly, *Pieris rapae*. Plant Ecology 189: 117-126.

Harvey JA, van Dam NM, Raaijmakers CE, Bullock JM, Gols R (2011) Tri-trophic effects of inter- and intra-population variation in defence chemistry of wild cabbage (*Brassica oleracea*). Oecologia 166:421-431.

Heil M, Ton J (2008) Long-distance signalling in plant defence. Trends in Plant Science 13: 264-272.

Hopkins RJ, van Dam NM, van Loon JJA (2009) Role of glucosinolates in insect-plant relationships and multitrophic interactions. Annual Review of Entomology 54: 57-83.

Inbar M, Doostdar H, Mayer RT (1999) Effects of sessile whitefly nymphs (Homoptera: Aleyrodidae) on leaf-chewing larvae (Lepidoptera: Noctuidae). Environmental Entomology 28: 353-357.

Kaplan I, Denno RF (2007) Interspecific interactions in phytophagous insects revisited: a quantitative assessment of competition theory. Ecology Letters 10: 977-994.

Karban R, Baldwin IT (1997) Induced responses to herbivory. Chicago: Chicago Univ. Press.

Kessler A, Baldwin IT (2004) Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco *Nicotiana attenuata*. The Plant Journal 38: 639-649.

Koornneef A, Leon-Reyes A, Ritsema T, Verhage A, Den Otter FC, Van Loon LC, Pieterse CM (2008) Kinetics of salicylate-mediated suppression of jasmonate signaling reveal a role for redox modulation. Plant Physiology 147: 1358-1368.

Kugimiya S, Shimoda T, Mcneil JN, Takabayashi J (2010) Females of *Cotesia vestalis*, a parasitoid of diamondback moth larvae, learn to recognise cues from aphid-infested plants to exploit honeydew. Ecological Entomology 35: 538-541.

Kusnierczyk A, Winge P, Jorstad TS, Troczynska J, Rossiter JT, Bones AM (2008) Towards global understanding of plant defence against aphids--timing and dynamics of early *Arabidopsis* defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. Plant, Cell and Environment 31: 1097-1115.

Lambdon PW, Hassall M, Boar RR, Mithen R (2003) Asynchrony in the nitrogen and glucosinolate leaf-age profiles of *Brassica*: is this a defensive strategy against generalist herbivores? Agriculture, Ecosystems & Environment 97: 205-214.

Lynch ME, Kaplan I, Dively GP, Denno RF (2006) Host-plant-mediated competition via induced resistance: interactions between pest herbivores on potatoes. Ecological Applications 16: 855-864.

Mayer RT, Inbar M, McKenzie CL, Shatters R, Borowicz V, Albrecht U, Powell CA, Doostdar H (2002) Multitrophic interactions of the silverleaf whitefly, host plants, competing herbivores, and phytopathogens. Archives of Insect Biochemistry and Physiology 51: 151-169.

Moyes CL, Raybould AF (2001) The role of spatial scale and intraspecific variation in secondary chemistry in host-plant location by *Ceutorhynchus assimilis* (Coleoptera: Curculionidae). Proceedings of the Royal Society B: Biological Sciences 268: 1567-1573.

Moyes CL, Collin HA, Britton G, Raybould AE (2000) Glucosinolates and differential herbivory in wild populations of *Brassica oleracea*. Journal of Chemical Ecology 26: 2625-2641.

Newton E, Bullock JM, Hodgson D (2009a) Bottom-up effects of glucosinolate variation on aphid colony dynamics in wild cabbage populations. Ecological Entomology 34: 614-623.

Newton EL, Bullock JM, Hodgson DJ (2009b) Glucosinolate polymorphism in wild cabbage (*Brassica oleracea*) influences the structure of herbivore communities. Oecologia 160: 63-76.

Ohgushi T (2005) Indirect interaction webs: Herbivore-induced effects through trait change in plants. Annual Review of Ecology, Evolution and Systematics 36: 81-105.

Ohgushi T, Craig TP, Price PW (2007) Ecological communities: plant mediation in indirect interaction webs. Cambridge University Press

Pieterse CMJ, Dicke M (2007) Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends in Plant Science 12: 564-569.

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal modulation of plant immunity. Annual Review of Cell and Developmental Biology 28: 489–521.

Poelman EH, Broekgaarden C, Van Loon JJA, 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 EH, Van Loon JJA, Van Dam NM, Vet LEM, Dicke M (2010) Herbivore-induced plant responses in *Brassica oleracea* prevail over effects of constitutive resistance and result in enhanced herbivore attack. Ecological Entomology 35: 240-247.

Poelman EH, Gols R, Snoeren TA, Muru D, Smid HM, Dicke M (2011a) Indirect plant-mediated interactions among parasitoid larvae. Ecology Letters 14: 670-676.

Poelman EH, Zheng SJ, Zhang Z, Heemskerk NM, Cortesero AM, Dicke M (2011b) Parasitoid-specific induction of plant responses to parasitized herbivores affects colonization by subsequent herbivores. Proceedings of the National Academy of Sciences USA 108: 19647–19652.

Ponzio C, Gols R, Pieterse CMJ, Dicke M (2013) Ecological and phytohormonal aspects of plant volatile emission in response to single and dual infestations with herbivores and phytopathogens. Functional Ecology 27: 587-598.

Ratzka A, Vogel H, Kliebenstein DJ, Mitchell-Olds T, Kroymann J (2002) Disarming the mustard oil bomb. Proceedings of the National Academy of Sciences USA 99: 11223-11228.

Rodriguez-Saona C, Chalmers JA, Raj S, Thaler JS (2005) Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. Oecologia 143: 566-577.

Rodriguez-Saona CR, Musser RO, Vogel H, Hum-Musser SM, Thaler JS (2010) Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. Journal of Chemical Ecology 36: 1043-1057.

Sandstrom J, Telang A, Moran NA (2000) Nutritional enhancement of host plants by aphids - a comparison of three aphid species on grasses. Journal of Insect Physiology 46: 33-40.

Schoonhoven LM, Van Loon JJA, Dicke M (2005) Insect-plant biology, 2nd edn. Oxford University Press, Oxford, UK.

Schramm K, Vassao DG, Reichelt M, Gershenzon J, Wittstock U (2012) Metabolism of glucosinolate-derived isothiocyanates to glutathione conjugates in generalist lepidopteran herbivores. Insect Biochemistry and Molecular Biology 42: 174-182.

Soler R, Badenes-Perez FR, Broekgaarden C, Zheng SJ, David A, Boland W, Dicke M (2012) Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. Functional Ecology 26: 156-166.

Stout MJ, Workman KV, Bostock RM, Duffey SS (1997) Specificity of induced resistance in the tomato, *Lycopersicon esculentum*. Oecologia 113: 74-81.

Stratmann JW (2003) Long distance run in the wound response - jasmonic acid is pulling ahead. Trends in Plant Science 8: 247-250.

Thompson GA, Goggin FL (2006) Transcriptomics and functional genomics of plant defence induction by phloem-feeding insects. Journal of Experimental Botany 57: 755-766.

Voelckel C, Baldwin IT (2004) Generalist and specialist lepidopteran larvae elicit different transcriptional responses in *Nicotiana attenuata*, which correlate with larval FAC profiles. Ecology Letters 7: 770-775.

Winde I, Wittstock U (2011) Insect herbivore counteradaptations to the plant glucosinolate-myrosinase system. Phytochemistry 72: 1566-1575.

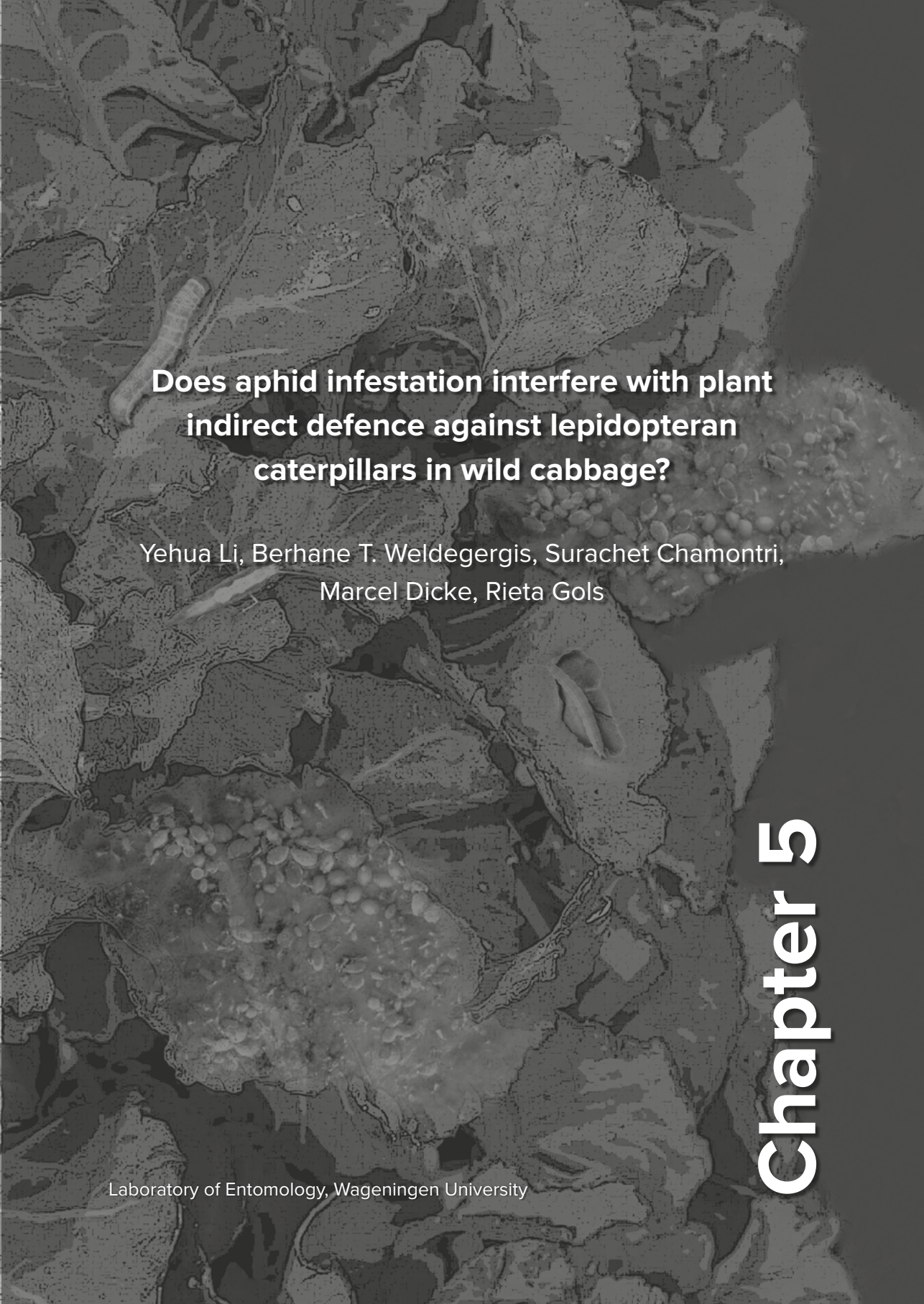
Zarate SI, Kempema LA, Walling LL (2007) Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiology 143: 866-875.

Zehnder CB, Hunter MD (2007) Interspecific variation within the genus *Asclepias* in response to herbivory by a phloem-feeding insect herbivore. Journal of Chemical Ecology 33: 2044-2053.

Zeier J (2005) Age-dependent variations of local and systemic defence responses in *Arabidopsis* leaves towards an avirulent strain of *Pseudomonas syringae*. Physiological and Molecular Plant Pathology 66: 30-39.

Zhang P, Broekgaarden C, Zheng S, Snoeren TAL, Van Loon JJA, Gols R, Dicke M (2013) Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. New Phytologist 197: 1291-1299.

Zhang PJ, Zheng SJ, Van Loon JJA, Boland W, David A, Mumm R, Dicke M (2009) Whiteflies interfere with indirect plant defense against spider mites in Lima bean. Proceedings of the National Academy of Sciences USA 106: 21202-21207.



# Does aphid infestation interfere with plant indirect defence against lepidopteran caterpillars in wild cabbage?

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Chapter 5

## Abstract

The effects of multiple herbivory on attraction of parasitoids to herbivore-induced plant volatiles (HIPV) is likely to depend on the specific combinations of attacking herbivore species, especially when their feeding modes activate different phytohormonal signalling pathways. We hypothesize that aphid infestation, activating salicylic acid signalling, may interfere with caterpillar-induced volatile production regulated by jasmonic acid signalling, through the antagonistic interaction between the two defence signalling pathways. We studied the effect of non-host aphid (*Brevicoryne brassicae*) infestation on the attraction of the parasitic wasps *Diadegma semiclausum* and *Microplitis mediator* to wild cabbage (*Brassica oleracea*) plants dually infested and infested with their respective hosts alone. *Diadegma semiclausum* preferred dually-infested plants with aphids and host caterpillars (*Plutella xylostella*) over plants infested by hosts only when aphid infestation had lasted 7 days, while the preference was reversed when aphid infestation was extended to 14 days. *Microplitis mediator* consistently preferred the plants infested by non-host aphids plus host caterpillars (*Mamestra brassicae*), regardless of aphid infestation period. Chemical analysis of the HIPV blends of plants dually infested and those infested with caterpillars alone, revealed that dual infestation consistently reduced the emission rates of various plant volatiles when plants were infested with *M. brassicae*, whereas this was only the case for plants infested with *P. xylostella* after extended aphid feeding. These results show that parasitoids are flexible in extracting information from these blends revealing host-presence, but also that the interactive effect of aphids and hosts is dynamic (effect of aphid-infestation duration) and can be host-parasitoid complex specific.

**Keywords:** indirect defence, natural enemies, multiple herbivory, parasitoid behaviour, volatile chemistry

## Introduction

Natural enemies of herbivorous insects have to find their hosts or prey in natural vegetations that are often structurally and chemically heterogeneous (Schoonhoven et al., 2005; Meiners, 2015). Plant volatiles are particularly important during foraging behaviour of natural enemies of insect herbivores (Heil, 2008; Dicke and Baldwin, 2010; Hare, 2011). Plant volatiles that are produced in response to herbivory, also referred to as herbivore-induced plant volatiles (HIPV), can be reliable cues in host location behaviour by parasitoids of insect herbivores. Moreover, HIPV may play an important role in structuring plant-associated insect communities as they mediate multitrophic interactions between plants, herbivores and their carnivorous natural enemies (Vet and Dicke, 1992; Dicke and Baldwin, 2010; Hare, 2011; Poelman et al., 2012).

The role of HIPV in attracting natural enemies of insect herbivores, has been well-studied in various plant-herbivore-carnivore systems (Mumm and Dicke, 2010). Initially, these studies focussed on infestation by single herbivore species. As herbivory by multiple species is the norm in nature, the effects of attack by more than one herbivore species are now receiving more attention in studies on volatile-mediated foraging by parasitoids of insect herbivores (Dicke, 2009; Dicke and Baldwin, 2010; De Rijk et al., 2013).

Plant volatile biosynthesis in response to insect herbivory is initiated by a combination of mechanical damage and elicitors from oral secretions of the herbivores (Turlings et al., 1990; Dicke, 2009; Mumm and Dicke, 2010; Bonaventure et al., 2011). The composition of the HIPV blend depends on the identity of the attacking herbivore and is also highly plant-species specific (Turlings et al., 1998; Dicke et al., 2003; Agbogba and Powell, 2007), although many of the blend components are produced by a wide range of plants (Mumm and Dicke, 2010). Different herbivore species attacking the same host plant may induce subtle differences in volatile profiles, even when the herbivores belong to the same feeding guild (De Moraes et al., 1998; Turlings et al., 1998; Dicke et al., 2003; Delphia et al., 2007). Moreover, multiple herbivory has been shown to alter HIPV emission (Rodriguez-Saona et al., 2003; Soler et al., 2007; Zhang et al., 2009). The effect of multiple herbivory on the behavioural response of parasitoids to HIPV depends on the specific combination of host and non-host herbivores (Rodriguez-Saona et al., 2003; Takabayashi et al., 2006; Dicke, 2009; Zhang et al., 2009; Erb et al., 2010; De Rijk et al., 2013; Zhang et al., 2013).

The production of HIPV is controlled by different plant signal-transduction pathways. The main signal-transduction pathways underlying plant indirect defences are the octadecanoid pathway with its key phytohormone jasmonic acid (JA) and the shikimic acid pathway with its key phytohormone salicylic acid (SA) (Kessler and Baldwin, 2001; Kessler and Baldwin, 2002; Dicke et al., 2009). Different types of attack often trigger different signalling pathways in the plant (Dicke et al., 2009); piercing-sucking insect herbivores such as aphids, mainly

activate SA-dependent responses, whereas leaf-chewing herbivores, like lepidopteran caterpillars, predominately trigger JA-dependent responses (Moran and Thompson, 2001; Zarate et al., 2007). Moreover, these two signalling pathways do not function independently, but they interact through cross talk. For example, SA has been shown to be an antagonist of JA defence signalling and vice versa when two herbivores triggering JA and SA, respectively, attack the same plant (Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Thaler et al., 2012). Although JA has been reported to be the most important regulator underlying induction of HIPV when plants are attacked by chewing herbivores, SA is also involved here (Ozawa et al., 2000; Engelberth et al., 2001; Wei et al., 2011). Furthermore, recent studies have shown that JA and SA signalling pathways do not only interact antagonistically but also synergistically (Engelberth et al., 2001; Howe and Jander, 2008), and can either positively or negatively affect the volatile emission or parasitoid attraction of plants with multiple infestation. For example, infestation with phloem-feeding whiteflies (*Bemisia tabaci*) was shown to perturb the JA-mediated plant volatile emission in response to leaf chewing herbivory (Rodriguez-Saona et al., 2003; Zhang et al., 2013). However, the parasitoid *Cotesia marginiventris* showed enhanced attraction to plants simultaneously infested by aphids (*Macrosiphum euphorbiae*) and caterpillars (*Spodoptera exigua*), compared to plants only infested with caterpillars (Rodriguez-Saona et al., 2005).

The temporal dynamics of induction of the signal-transduction pathways regulating HIPV emission differ (Dicke, 2009; Dicke et al., 2009). Metabolic changes in plants in response to chewing herbivores, including the emission of HIPV generally occur within hours to days after feeding damage (Kunert et al., 2002; Dicke et al., 2009), whereas the response of plants to the more subtle damage caused by aphid feeding, often takes much more time (Moran and Thompson, 2001; Schmelz et al., 2003; Guerrieri and Digilio, 2008; Dicke, 2009). Poelman *et al.* (2008) reported that at the molecular level initial herbivory affected the transcriptional response of cultivars of white cabbage to a second herbivore and that the magnitude of expression differed between the plant cultivars used in the study. The specific temporal dynamics of defence pathway induction may have consequences for the response of a plant to two herbivores that attack the plant separated in time. For instance, the suppression of JA-responsive gene expression and JA concentration in aphid-infested *Brassica oleracea* plants was only found to occur 6 days after aphid attack (Soler et al., 2012).

In addition to attacker-specific variation in HIPV production, there is also within-plant species variation in HIPV production when attacked by the same insect herbivore, which can affect the attraction of parasitoids (Lou et al., 2006; Gols et al., 2009; Poelman et al., 2013). Although variation in the production of HIPVs has been observed among cultivars of numerous crops, intra-specific variation in HIPV production among genotypes of wild species has been rarely studied (Hare, 2011). Significant quantitative and qualitative variation was observed among 12 genotypes of *Solanum carolinense*, with up to 10-fold variation in total HIPV production among lines (Casey M. Delphia et al., 2009). Wild cabbage

plants originating from three populations in Dorset, UK differ in their HIPV blends and attractiveness to the larval parasitoid *Cotesia rubecula*, a specialist parasitoid of *Pieris rapae* on brassicaceous plants (Gols et al., 2011).

The objective of this study is to investigate whether volatile-mediated foraging behaviour of two parasitoid species, *Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae) and *Microplitis mediator* Halliday (Hymenoptera: Braconidae) to wild cabbage plants infested by their respective hosts, *Plutella xylostella* L. (Lepidoptera: Plutellidae) and *Mamestra brassicae* L. (Lepidoptera: Noctuidae), is affected when the plants are also infested with aphids (*Brevicoryne brassicae* L., Hemiptera, Aphididae). To test for the effect of variation in secondary chemistry and the plant's response to herbivory, we used wild cabbage (*Brassica oleracea* L., Brassicacea) plants that originated from three wild populations in Dorset, UK, and are known do differ in volatile and non-volatile secondary chemistry (Gols et al., 2008; Gols et al., 2011). We compared the behavioural response of the two parasitoid species towards volatiles emitted by plants infested with hosts and aphids and plants infested with hosts alone in a Y-tube olfactometer. We varied the duration of initial aphid infestation before the host was introduced. We hypothesize that aphid infestation and as a result of this, their density, would interfere with the caterpillar-induced volatile production through the antagonistic interaction between plant SA and JA signalling pathways. In addition, we collected and analysed the volatiles emitted by the plants exposed to the various aphid and caterpillar treatments to assess the underlying chemical basis of the observed behavioural responses.

## Material and Methods



### Plants and insects

Seeds of three wild cabbage (*Brassica oleracea*) populations originated from sites known as Winspit (50°35'N, 2°02'W), Old Harry (50°38'N, 1°55'W) and Kimmeridge (50°36'N, 2°07'W), hereafter abbreviated to WIN, OH and KIM, respectively. Seeds were sown one week before transplanting seedlings into individual pots (1.5 l) containing potting soil (Lentse potgrond no. 4; Lent, The Netherlands). Plants were grown in a greenhouse at 22 ± 3 °C, 50–70 % relative humidity [RH], and a light:dark regime [L:D] of 16:8 h. Plants were watered every two days until they were three weeks old, and watered daily hereafter.

All insects were originally collected from cabbage fields in the vicinity of Wageningen University and all cultures were maintained on Brussels sprouts plants (*B. oleracea* L. var. gemmifera cv. Cyrus) in a greenhouse or a climate room at 22 ± 2˚C, 60-70% RH and 16:8 h L:D photo regime.

*Diadegma semiclausum* is a specialist larval endoparasitoid that can develop in all four larval stages of its host, *Plutella xylostella*. *Microplitis mediator* is a generalist larval endoparasitoid that can attack larval stages of several noctuid species including *Mamestra brassicae*. Female *M. mediator* can parasitize first-to-third larval instars of *M. brassicae*. The two parasitoid species were reared on plants infested with host caterpillars until the parasitoids had completed their immature development and pupated. Newly emerged *D. semiclausum* and *M. mediator* adults were collected and transferred to clean insect cages and allowed to mate. Adult wasps were kept in a climate cabinet at 25 ± 1 ˚C, and L16:D8 photo regime and were provided with honey and 6-10% sugar water as a food source. Both wasp species were considered naïve as plant material had been removed from the cage before the wasps eclosed. Female wasps that were used in the experiments were 2-6 (*D. semiclausum*) or 3-7 (*M. mediator*) days old, and did not have oviposition experience.

### Plant treatment

When plants were four weeks old, twenty adult aphids (*B. brassicae*) were introduced on the first fully expanded leaf of plants from the three populations and were allowed to feed and reproduce for 7 or 14 days prior to testing in a Y-tube olfactometer (see below). The infested plants were covered with nylon nets (48 × 60 cm, Bugdorm, Taiwan) to prevent cross-contamination. Approximately 24 hours before the behavioural experiments, 10 *P. xylostella* caterpillars (L2) or 30 one-day-old *M. brassicae* caterpillars were introduced on the same leaf as where the aphids had been introduced or on a comparable leaf when plants only received a host caterpillar treatment. Insects remained on the plant when used in the bioassay.



### Bioassay

The experiment was conducted in a laboratory at 22±2 ˚C in a Y-tube olfactometer (Steinberg et al., 1992), which was supplied with fluorescent lights from above with an intensity of 30-35 µmol photons / $m^2$ / s. Purified air (filtered through activated charcoal) was led at 2 l / min into each of the two glass vessels (35 l) containing an odour source (intact plants) which were connected to the arms of the Y-tube olfactometer (diameter 3.5 cm, stem length 20 cm, arm length 10 cm). The experiment started with the release of a wasp at the base of the Y-tube. Each wasp was observed for maximally 10 min, and a choice was recorded when the wasp reached the middle of either arm and remained in that arm for at least 15 s. When the wasp did not make a choice within 10 min, a 'no choice' behavioural response was recorded. Each wasp was used only once. One experimental replicate consisted of one pair of cabbage plants tested with 8-10 responding female parasitoids.

First, we conducted a pilot test to examine whether both wasp species discriminated between host-infested (24h) and uninfested plants. This experiment was conducted with WIN plants only and was repeated five times with both wasp species.

We then continued with the main experiment in which we compared the response of both parasitoid species to plants infested with hosts alone and plants dually infested with hosts and aphids. The pair-wise comparisons were conducted with plants from each of the three plant populations and with each of the two aphid infestation periods, 7 or 14 days, resulting in a total of six pair-wise comparisons with each parasitoid species. Eight experimental replicates were carried out per combination of cabbage population and aphid-infestation period. After testing the response of the parasitoids to the plants in the Y-tube olfactometer, the number of aphids on dually-infested plants was counted, and the area of leaf tissue consumed by caterpillars was quantified using a transparent plastic sheet with a 1-mm$^2$ grid. The bioassays with the two parasitoid species and various pair-wise plant combinations were randomized over the experimental period of three months.

### Headspace collection

Volatiles were collected from 18 population-herbivore treatment combinations that were tested in the Y-tube olfactometer with the exception of uninfested control plants. Plants were grown, inoculated with insects and incubated as described in the 'plant treatment' section. Volatiles were collected from individual plants (n=8) using a dynamic headspace collection system in a climate room (21±2°C, RH 60–70%). The pot containing the plant was wrapped in aluminium foil and the plant was transferred to a clean 35-l glass collection container. Before sampling, the container was purged for 30 minutes at 220 ml / min. Compressed air was filtered through activated charcoal before entering the glass container with the plant. Subsequently, volatiles were collected by drawing air from the container through a stainless steel cartridge filled with 200 mg Tenax TA (20/35 mesh; CAMSCO, Houston, TX, USA) for 2 hours at a flow of 200 ml / min using an external pump. Plants of each treatment and cabbage population were randomly selected for volatile trapping on each experimental day. Volatiles were also collected (n= 12) from pots containing soil only, that were wrapped in aluminium foil. Volatile compounds detected in these control samples were excluded from the data obtained for the plant samples. The above-ground part of each plant was weighed immediately after volatile trapping. The cartridges filled with Tenax with the trapped headspace samples were dry-purged for 15 min with a nitrogen ($N_2$) flow at 50 ml / min and stored at ambient temperature.

### Chemical analysis of volatiles

Separation and identification of plant volatiles was carried out using Thermo Trace Ultra gas chromatography (GC) combined with Thermo Trace DSQ quadrupole mass spectrometer (MS), both from Thermo (Thermo Fisher Scientific, Waltham, USA). The volatiles were thermally released from the Tenax TA adsorbent (Ultra 50:50 thermal desorption unit, Markes, Llantrisant, UK) at 250 °C for 10 min under a helium flow of 20 ml / min, while simultaneously re-collecting the volatiles in a thermally cooled universal solvent trap (Unity, Markes) at 0 °C.

Once the desorption process was completed, volatile compounds were released from the cold trap by ballistic heating at 40 °C / s to 280 °C and this temperature was maintained for 10 min, while the volatiles being transferred to a ZB-5MSi analytical column with 30 m x 0.25 mm I.D. x 0.25 µm F.T. dimensions and 5 m built-in guard column (Phenomenex, Torrance, CA, USA), in a splitless mode for further separation. The GC oven initial temperature was set to 40 °C and held for 2 min, which was then raised at 6 °C / min to a final temperature of 280 °C, where it was kept for 4 min under a constant helium flow of 1 ml / min. The DSQ MS was operated in a scan mode with 35 - 400 amu mass range at 4.70 scans $s^{-1}$ and spectra were recorded in electron impact ionisation (EI) mode at 70 eV. MS transfer line and ion source were set to 275 and 250 °C, respectively. Tentative identification of compounds was based on comparison of mass spectra with those in the NIST 2005 and Wageningen Mass Spectral Database of Natural Products MS libraries as well as experimentally obtained linear retention indices (LRI).

### Statistics

Of the 1044 tested parasitoids, 93.2% made a choice in the Y-tube olfactometer. The non-responding individuals were excluded from the data analysis. The response variable for the statistical analysis was the percentage of the total number of responding parasitoids choosing dually-infested plants in each experimental replicate. A general linear model (GLM) was used to compare the mean preference percentages among the data groups. Here, aphid infestation period (7 or 14 days), cabbage population (KIM, OH and WIN), and their interaction term were entered as fixed factors in the model. For some contrasts specified in the results section, one-sample t-tests were used to determine whether the overall distribution of the wasps over the odour sources deviated from 50:50 ($H_0$: µ=50%). The number of aphids counted at 7 or 14 days after inoculation was compared on the three cabbage populations using one-way ANOVA. Leaf damage areas were compared between plants with a single infestation of caterpillars and plants with a dual infestation of both caterpillars and aphids in two-sample t-tests. All statistical tests were performed in SPSS (IBM SPSS statistics version 22).

The volatile emission patterns, quantified as peak areas of compounds divided by the fresh mass of the plants, were analysed through multivariate data analysis using OPLS-DA (orthogonal projection to latent structures discriminant analysis). The analysis determines if samples belonging to the different treatment groups (singly- and dually-infested plants) can be separated on the basis of quantitative and qualitative differences in their volatile blends. A Y-data matrix of dummy variables was included, assigning a sample to its respective class. The SIMCA-P+ 14.0 software program (UmetricsAB, Umeå, Sweden) then approximates the point 'swarm' in X (matrix with volatile compounds) and Y in components in such a way that maximum covariation between the components in X and Y is achieved (Eriksson et al., 2013). OPLS-DA further uses information in the Y matrix to decompose the X matrix into blocks of structured variation correlated to and orthogonal to Y, respectively, to separate

predictive from the non-predictive (orthogonal) variation (Bylesjö et al., 2006). Data were log-transformed, mean-centred and scaled to unit variance before they were subjected to the analysis. In the analysis, we did the pairwise comparisons on the volatile blends of plants with host caterpillars (either *P. xylostella* or *M. brassicae*) only and plants with both host caterpillars and aphids for each cabbage population separately. The parameter $Q^2$ in SIMCA is commonly used in the validation of OPLS-DA models; it indicates the predictive ability of the method, and $Q^2 > 0.5$ is an indicator for good predictability (Eriksson et al., 2013). Therefore, when the $Q^2$ value is larger than 0.5 and at least one significant principal component (PC) was detected, the model is considered to be significant in the discriminant analyses. The chemical compounds that had a VIP (variable importance in the projection) value higher than 1 were the most important compounds in terms of differentiating odour blends (Eriksson et al., 2013), and are presented in bold in Table 2. Per plant population-caterpillar species combination, we also compared the number of compounds emitted by the dually-infested plants of which the emission levels increased and decreased relative to the levels emitted by plant infested with caterpillar alone using $\chi^2$-tests.

## Results

### Preference of *D. semiclausum* and *M. mediator* when offered volatiles from host-infested versus uninfested plants

In the dual-choice bioassay where wasps were given the choice between a host-infested and an un-infested plant, both *D. semiclausum* and *M. mediator* significantly preferred volatiles from plants infested by host caterpillars over volatiles from uninfested plants (Fig 1, t-test, *D. semiclausum* $t_4 = 15.0$, $P = 0.001$; *M. mediator* $t_4 = 2.95$, $P = 0.042$). On average 87.5% of the *D. semiclausum* females and 71.7% of *M. mediator* females made a choice for the volatiles emitted by host-infested plants.

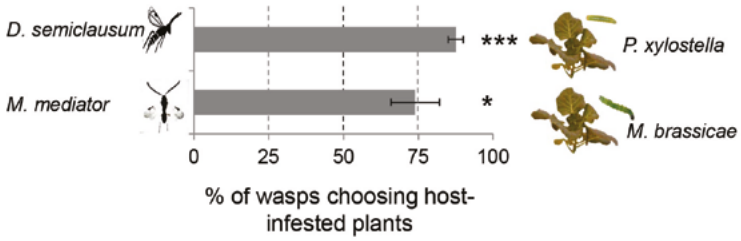


**Figure 1.** Mean attraction percentage (± SE) of *Diadegma semiclausum* (top bar) and *Microplitis mediator* wasps (bottom bar) to volatiles from WIN plants (*Brassica oleracea*) infested with their respective host caterpillars for 24h, when the alternative volatile source is an uninfested WIN plant. Host-infested plants were challenged with 10 *P. xylostella* caterpillars (L2) in the tests with *D. semiclausum* or 30 *M. brassicae* caterpillars (L1) in the tests with *M. mediator*. Asterisks (* $0.01 < P \leq 0.05$, *** $P \leq 0.001$) indicate a preference that is significantly different from a 50:50 distribution based on a one-sample t-test.

### Preference of *D. semiclausum* and *M. mediator* when offered volatiles from plants with single versus dual infestation

When offered volatiles from plants with single and dual infestation, the preference of *D. semiclausum* was consistent across the plant populations ($F_{2,42}$ = 2.15, $P$ = 0.13), but differed significantly between plants co-infested with aphids for 7 or 14 days ($F_{1,42}$ = 12.2, $P$ < 0.001). The interaction term between population and infestation duration was not significant ($F_{2,42}$ = 0.38, $P$ = 0.69). *Diadegma semiclausum* preferred volatiles from *P. xylostella*-infested plans that were infested with aphids for 7 days over plants infested with *P. xylostella* only (Fig 2A; t-test, $t_{23}$ = 2.30, $P$= 0.03). In contrast, when plants had been infested with aphids for 14 days, *D. semiclausum* preferred volatiles emitted by plants that had been infested with only hosts (Fig 2B; t-test; $t_{23}$= 2.66, $P$= 0.014).

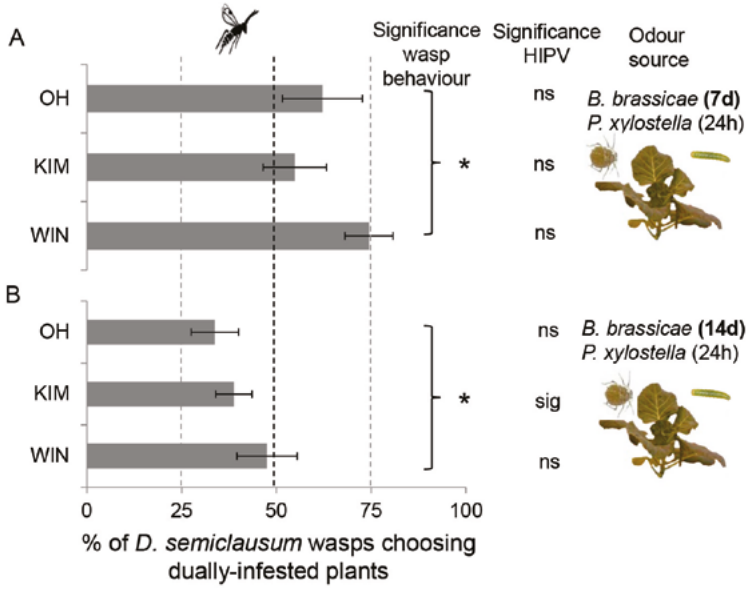


**Figure 2.** Mean percentage (± SE) of *Diadegma semiclausum* wasps choosing plants of three different cabbage (*Brassica oleracea*) populations (OH, KIM, WIN) infested with hosts (*Plutella xylostella*) and aphids (*Brevicoryne brassicae*) when the alternative volatile source is a plant from the same population infested with only hosts. Dually-infested plants were challenged with 20 adult aphids for 7 days (A) or 14 days (B) and 10 *P. xylostella* caterpillars (L2) for 24 hours, whereas the singly-infested plants were only challenged by 10 *P. xylostella* caterpillars for 24 hours. Asterisk indicates whether there is a significant preference for one of the two odour sources using one-sample t-test (μ=50%): * 0.01 < $P$ ≤ 0.05. The significance levels of pair-wise PLS-DA models on HIPV compounds emitted by singly- dually-infested plants are also given in the graph (ns= not significant; sig= significant).

The preference of *M. mediator* for volatiles from plants with single or dual infestation was not affected by cabbage population ($F_{2,42}$ = 1.10, $P$ = 0.34), nor by the duration of aphid infestation ($F_{1,42}$ = <0.001, $P$ = 1.00), or heir interaction term ($F_{2,42}$ = 0.47, $P$ = 0.63). Overall,

*M. mediator* was more attracted to volatiles from plants infested with hosts plus aphids than to plants infested with hosts alone at both aphid infestation periods, regardless of the cabbage populations (Fig 3; t-test; $t_{23}$= 26.4, $P$< 0.001).

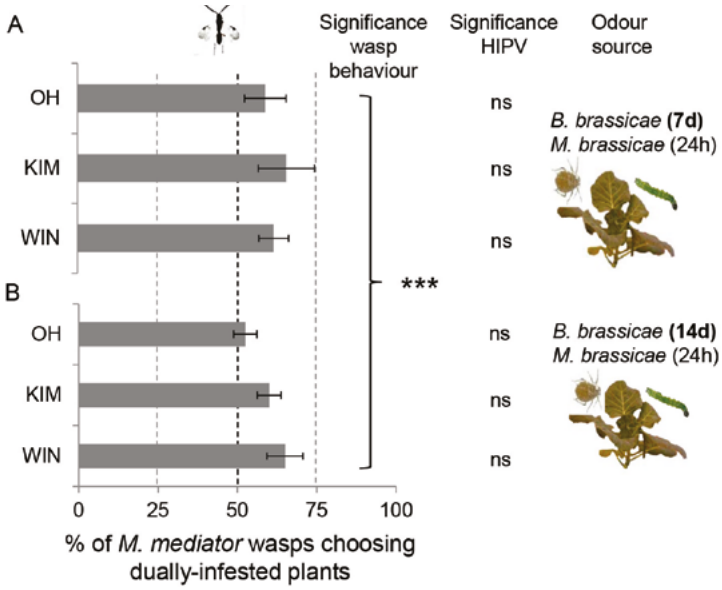


**Figure 3.** Mean attraction percentage (± SE) of *Microplitis mediator* wasps to plants of three different cabbage (*Brassica oleracea*) populations (OH, KIM, WIN) infested with hosts (*Mamestra brassicae*) and aphids (*Brevicoryne brassicae*) when the alternative volatile source is a plant from the same population infested with only hosts. Dually-infested plants were challenged with 20 adult aphids for 7 days (A) or 14 days (B) and 30 *M. brassicae* caterpillars (L1, one-day-old) for approximately 24 hours, whereas the singly-infested plants were only challenged by 30 *M. brassicae* caterpillars for 24 hours. Asterisks indicate whether there is a significant preference for one of the two odour sources using one-sample t-test (μ=50%): *** $P$ ≤0.001. The significance levels of pair-wise PLS-DA models on HIPV compounds emitted by singly- dually-infested plants are also given in the graph (ns=not significant).



### Aphid numbers and caterpillar leaf consumption

The mean numbers of aphids did not statistically differ among the three cabbage populations, neither on plants infested with aphids for 7 days nor for 14 days for both caterpillar species (One-way ANOVA population effect, 7 days of aphid infestation plus *P. xylostella*, $F_{(2, 21)}$= 0.27, $P$= 0.77; 14 days aphid infestation plus *P. xylostella*, $F_{(2, 21)}$= 0.86, $P$= 0.44; 7 days of aphid infestation plus *M. brassicae*, $F_{(2, 21)}$= 1.1, $P$= 0.35; 14 days of aphid infestation plus *M. brassicae*, $F_{(2, 21)}$= 2.7, $P$= 0.094). The overall mean (±SE) number of aphids on plants of the three cabbage populations was 160.5 ± 4.2 after 7 days and 427.1 ± 9.5 after 14 days of aphid infestation.

The amount of leaf damage caused by *P. xylostella* caterpillars did not statistically differ between plants with or without aphids (t- test; $t_{26}$= 0.46, $P$= 0.65), although overall

feeding damage tended to be higher on WIN than on KIM and OH plants, but this was not statistically significant (ANOVA population effect, $F_{(2, 25)}$= 3.39, $P$= 0.051). On average, ten L2 *P. xylostella* caterpillars consumed 27.9 ± 1.5 mm² of leaf tissue in 24 h. There was also no significant difference between the leaf areas consumed by *M. brassicae* caterpillars on plants with or without aphids (two-sample t-test; $t_{44}$= 0.056, $P$= 0.96), and the leaf damage did not differ among the three cabbage populations (ANOVA, $F_{(2, 43)}$= 2.07, $P$= 0.139). Thirty L1 *M. brassicae* caterpillars consumed on average 81.3 ± 3.1 mm² of leaf tissues in 24h.

**Volatile analysis**

A total of 51 different volatile compounds were detected in the headspace of the three cabbage populations, across all treatments (Table 1). Overall, all plants emitted the same compounds but amounts of various compounds varied among treatments (Table 1). Pair-wise OPLS-DA of volatiles emitted by plants infested with hosts alone versus dually-infested plants showed poor separation of the volatile blends of the two groups for each of the plant populations infested with either caterpillar species. None of the OPLS-DA models were significant after 7 days of aphid infestation and the model was significant only for *P. xylostella*-infested KIM plants after 14 days of aphid infestation (Fig 2, Appendix 1).

Many compounds were emitted at lower levels by plants infested with caterpillars and aphids than by plants infested with caterpillars alone. To determine whether this was significant, we compared the number of compounds that were emitted at higher and lower levels compared to the emission of these compounds from plants infested with caterpillars alone using $\chi^2$-tests. In OH and WIN plants infested with *P. xylostella* plus aphids, the number of compounds of which the emission decreased compared to caterpillar-only infestation was significantly higher than the number of compounds of which the emission increased, but only when the aphids were on the plants for 14 days (Table 1a). KIM plants co-infested with aphids for 7 days, emitted significantly more compounds in higher than in lower rates compared to plants infested with caterpillars alone, whereas the number of compounds of which the emission increased and decreased was similar after 14 days of aphid infestation (Table 1a).

In each of the three populations, plants infested with *M. brassicae* plus aphids emitted more compounds at lower than at higher levels relative to the levels emitted by plants infested with *M. brassicae* alone. This was the case for both aphid infestation periods, except for OH plants co-infested with aphids for 7 days where the number of compounds with increased and decreased emission levels was similar (Table 1b).

**Table 1** Volatile emissions (mean ± SE, n= 6-8) by plants of three wild cabbage populations (*Brassica oleracea*), OH, KIM, and WIN, in response to feeding by two caterpillar species (A) *Plutella xylostella* or (B) *Mamestra brassicae* that were either feeding alone or together with aphids (*Brevicoryne brassicae*) for 7 or 14 days. Volatile compounds emitted by dually-infested plants in bold numbers are those that contributed the most (VIP value > 1) to the separation of blends from host-infested plants of the same population based on OPLS-DA multivariate statistics. Volatile emissions are given as mean chromatogram peak area ± SE per g fresh weight of foliage divided by $10^4$.

A)

| Compound | OH | | | KIM | | | WIN | | |
|---|---|---|---|---|---|---|---|---|---|
| | Px | Bb(7d)+Px | Bb(14d)+Px | Px | Bb(7d)+Px | Bb(14d)+Px | Px | Bb(7d)+Px | Bb(14d)+Px |
| **Alcohols** | | | | | | | | | |
| (*Z*)-3-Hexen-1-ol | 4.5±2.5 | **1.4±0.3** | **1.4±0.3** | 2.3±1.2 | 7.6±6.5 | **7.1±2.9** | 4.5±1.9 | 2.8±1.4 | **1.7±1.0** |
| 1-Octen-3-ol | 6.6±1.2 | 11.8±3.1 | **16.8±2.9** | 5.0±0.6 | 13.7±5.0 | **16±4.0** | 10.6±2.0 | 8.5±1.4 | 18.0±3.5 |
| **Esters** | | | | | | | | | |
| (*Z*)-2-Penten-1-ol, acetate | 1.5±0.4 | **0.80±0.16** | **0.7±0.2** | 2.1±1.6 | 3.7±3.0 | 2.3±1.1 | 2.3±1.2 | 0.9±0.2 | 0.31±0.10 |
| (*Z*)-3-Hexen-1-ol, acetate | 373±186 | **159±51** | **100±20** | 252±183 | 474±385 | **463±207** | 248±92 | 167±51 | **88±40** |
| Hexyl acetate | 3.5±1.3 | **1.8±0.8** | **1.3±0.3** | 2.0±0.8 | 5.0±3.8 | **7.8±3.6** | 2.4±0.6 | 1.5±0.4 | **0.60±0.16** |
| (*Z*)-3-Hexenyl butyrate | 0.8±0.7 | **0.08±0.03** | **0.17±0.06** | 0.10±0.05 | 0.16±0.11 | **0.7±0.4** | 0.6±0.3 | 0.35±0.15 | 0.7±0.4 |
| Methyl salicylate | 4.3±3.1 | **7.1±2.9** | 4.1±1.2 | 5.1±3.8 | 2.6±0.9 | 0.73±0.12 | 2.7±1.6 | 3.9±1.8 | **1.1±0.2** |
| (*Z*)-3-Hexen-1-ol, 2-methylbutanoate | 1.3±0.73 | **0.47±0.15** | **0.30±0.11** | 0.30±0.19 | 1.5±1.2 | **1.4±0.7** | 0.7±0.2 | 0.6±0.3 | **0.18±0.10** |
| (*Z*)-3-Hexen-1-ol, 3-methylbutanoate | 0.29±0.19 | **0.17±0.09** | **0.01±0.01** | 0.05±0.03 | 0.08±0.08 | **0.16±0.12** | 0.11±0.08 | 0.05±0.04 | 0.01±0.01 |
| Linalyl acetate | 0.8±0.3 | **1.1±0.3** | 0.67±0.14 | 2.6±1.7 | 2.5±1.7 | 1.4±0.5 | 1.1±0.4 | 4.0±3.0 | **0.42±0.13** |
| (*Z*)-4-tert-Butylcyclo-hexyl acetate | 1.9±0.5 | 1.7±0.5 | 1.5±0.3 | 3.8±1.9 | 2.8±1.6 | **1.7±0.6** | 2.4±0.4 | 2.7±1.4 | 1.4±0.4 |
| α-Terpinyl acetate | 0.18±0.06 | 0.21±0.06 | 0.11±0.03 | 0.37±0.15 | 0.26±0.08 | 0.21±0.11 | 0.21±0.05 | 0.23±0.10 | 0.11±0.03 |
| **Ketones** | | | | | | | | | |
| 3-Methyl-2-butanone | 1.1±0.8 | **0.13±0.11** | **0.05±0.04** | 1.5±0.5 | 2.4±0.9 | **0.35±0.14** | 1.8±0.7 | 0.7±0.4 | **0.6±0.3** |
| 3-Pentanone | 1.9±0.4 | **0.92±0.14** | 1.6±0.5 | 1.4±0.5 | 3.3±2.5 | **8.1±5.6** | 6.3±4.3 | 1.7±0.4 | 1.6±0.4 |
| 3-Methyl-2-pentanone | 6.4±3.5 | **1.7±0.9** | **0.41±0.08** | 3.9±1.1 | 3.5±1.2 | **1.4±0.5** | 7.4±4.4 | 2.8±0.8 | **0.81±0.19** |
| α-Isomethylionone | 0.40±0.17 | 1.4±0.7 | 0.45±0.11 | 1.5±0.8 | 1.2±0.6 | **0.6±0.2** | 1.5±0.7 | 1.1±0.6 | 0.28±0.05 |
| **S- & N- containing compounds** | | | | | | | | | |
| 3-Methylbutanenitrile | 1.8±0.6 | 2.0±0.8 | 0.9±0.2 | 2.4±1.5 | 0.6±0.3 | 0.51±0.13 | 0.3±0.2 | 3.4±2.4 | 0.25±0.08 |
| Dimethyl disulfide | 7.6±2.2 | **4.3±0.8** | 8.5±1.5 | 13.9±4.5 | 16±6.2 | **50±19** | 7.1±1.5 | 7.1±2.6 | 12.2±2.3 |
| Dimethyl trisulfide | 2.9±1.1 | 1.7±0.3 | 2.5±0.5 | 6.0±2.5 | 6.2±2.7 | 10.1±2.2 | 4.0±1.3 | 2.3±0.5 | 2.4±0.4 |
| 3-Butenyl isothiocyanate | 27.4±9.3 | 28±7.1 | 44±13 | 13.7±5.5 | 11±4.5 | 10.4±3.5 | 58±15 | 19.6±2.4 | **159±45** |
| Benzyl nitrile | 7.3±3.7 | **10.5±2.6** | 3.4±1.0 | 0.41±0.18 | 0.32±0.08 | 0.38±0.09 | 1.4±0.4 | 1.4±0.4 | 1.1±0.3 |
| Indole | 5.1±2.0 | **1.5±0.6** | **1.3±0.5** | 0.9±0.8 | 1.0±0.4 | 5.6±3.5 | 4.8±3.1 | 2.0±1.0 | **1.7±0.7** |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Terpenoids** | | | | | | | | | |
| α -Thujene | 273±32 | 294±44 | 223±47 | 69±44 | 135±56 | 60±29 | 284±57 | 302±33 | 298±78 |
| α -Pinene | 81.9±9.2 | 82±14 | 68±11 | 29±14 | 41±16 | 25±10 | 93±18 | 84.1±8.2 | **77±18** |
| Sabinene | 661±75 | 758±117 | **587±100** | 195±121 | 364±151 | 163±78 | 703±143 | 750±87 | 701±174 |
| β-Pinene | 66.1±6.6 | 64.1±9.8 | 53.7±9.0 | 19±10 | 28±11 | 21.0±9.4 | 65±13 | 67.8±7.2 | 58±14 |
| β-Myrcene | 198±23 | 213±32 | 179±31 | 59±31 | 104±43 | 49±22 | 216±42 | 227±26 | 218±56 |
| α -Terpinene | 25.0±6.4 | **20.5±3.3** | 24.5±7.7 | 5.8±3.3 | 11.4±6.0 | 4.7±2.3 | 25.9±6.7 | 27.3±5.9 | 27.1±9.8 |
| Limonene | 272±31 | 299±42 | 262±55 | 102±46 | 182±73 | 97±41 | 326±66 | 330±35 | **306±71** |
| 1,8-Cineole | 68.9±8.3 | 76±13 | 60±11 | 19±11 | 28±13 | 16.2±7.5 | 65±14 | 46.7±9.9 | 66±19 |
| (*E*)-β-Ocimene | 5.8±1.0 | 5.5±1.0 | 4.8±0.9 | 2.3±1.5 | 3.1±1.3 | 1.9±0.7 | 6.2±1.4 | 6.4±0.7 | 5.3±1.3 |
| γ-Terpinene | 17.2±4.4 | 15±2.4 | 16.7±5.1 | 4.2±2.2 | 8.0±4.1 | 3.5±1.6 | 17.8±4.6 | 19.3±4.1 | 19±6.7 |
| (*E*)-β-Terpineol | 2.4±0.3 | 2.8±0.5 | **1.5±0.3** | 0.6±0.4 | 1.2±0.5 | 0.7±0.3 | 2.6±0.6 | 2.4±0.4 | 2.4±0.7 |
| α-Terpinolene | 11.7±2.5 | 10.1±1.8 | 12±2.8 | 3.1±1.7 | 5.5±2.7 | 2.6±1.1 | 13.3±3.0 | 12.5±2.4 | 12.3±4.1 |
| Linalool | 1.8±0.5 | **2.1±0.3** | 1.3±0.3 | 6.1±5.2 | 2.2±1.0 | 3.2±1.8 | 3.1±1.6 | 5.7±2.9 | 2.9±0.8 |
| [1](*E*)-DMNT | 48±20 | 8.9±1.9 | 15.3±7.4 | 6.1±2.0 | 11.0±3.8 | 5.3±1.9 | 2.6±0.7 | 2.7±1.3 | **0.68±0.11** |
| Menthol | 0.51±0.18 | **0.40±0.07** | 0.5±0.3 | 2.9±2.5 | 1.8±1.3 | 0.5±0.2 | 1.0±0.5 | 1.6±1.2 | 0.17±0.07 |
| 4-Terpineol | 0.57±0.06 | 0.68±0.11 | 0.59±0.13 | 0.18±0.08 | 0.38±0.12 | 0.17±0.08 | 0.7±0.2 | 0.66±0.18 | 0.9±0.2 |
| α-Terpineol | 2.9±1.1 | **4.0±0.8** | 1.7±0.6 | 0.39±0.19 | 2.2±1.1 | **1.4±1.0** | 6.4±2.6 | 4.7±1.9 | 4.5±2.2 |
| β-Cubebene | 0.21±0.05 | **0.10±0.03** | 0.12±0.06 | 0.16±0.04 | 0.13±0.06 | 0.10±0.04 | 0.3±0.2 | 0.12±0.04 | **0.07±0.02** |
| β-Elemene | 0.46±0.43 | 0.9±0.6 | **4.8±2.3** | 1.3±0.9 | 7.2±5.7 | **6.3±3.1** | 8.6±4.3 | 1.5±0.8 | **0.5±0.3** |
| Bicyclosesquiphellandrene | 0.05±0.02 | 0.12±0.05 | **0.28±0.07** | 0.02±0.02 | 0.14±0.05 | **0.40±0.15** | 0.08±0.04 | 0.10±0.04 | 0.35±0.19 |
| (*Z*,*E*)-α-Farnesene | 0.8±0.3 | 0.65±0.18 | **0.41±0.12** | 0.08±0.03 | 0.16±0.07 | **0.8±0.2** | 0.5±0.2 | 0.5±0.2 | 0.20±0.07 |
| β-Chamigrene | 0.88±0.67 | **0.33±0.09** | 1.2±0.5 | 0.47±0.09 | 0.29±0.08 | **0.9±0.4** | 1.7±0.6 | 0.40±0.11 | 0.25±0.08 |
| (*E*,*E*)-α-Farnesene | 18.6±6.7 | 18.4±5.7 | 15.7±5.8 | 2.7±0.9 | 5.2±1.6 | **19±9.2** | 9.2±2.3 | 14.1±6.3 | 5.4±1.1 |
| Germacrene A | 0.7±0.7 | **0.15±0.08** | 0.9±0.4 | 0.24±0.14 | 0.16±0.06 | **0.9±0.5** | 1.2±0.6 | 0.18±0.08 | 0.06±0.05 |
| (*Z*)-α-Bisabolene | 0.22±0.2 | **0.02±0.02** | 0.28±0.14 | 0.07±0.04 | 0.04±0.02 | **0.25±0.15** | 0.4±0.2 | 0.05±0.03 | 0.02±0.01 |
| Nerolidol | 0.45±0.13 | 0.38±0.10 | 0.29±0.07 | 0.30±0.13 | 0.6±0.4 | 0.34±0.11 | 0.21±0.07 | 0.22±0.09 | **0.12±0.06** |
| **other or unknown** | | | | | | | | | |
| 4-Ethenyl cyclohexene | 3.4±2.4 | 2.7±1.8 | 2.6±1.1 | 1.9±1.1 | 5.2±3.3 | 1.0±0.3 | 2.8±1.4 | 2.5±1.8 | 1.1±0.6 |
| Unknown | 0.8±0.2 | 0.69±0.18 | 0.68±0.18 | 0.7±0.3 | 0.6±0.2 | 0.8±0.2 | 1.0±0.3 | 0.60±0.11 | **0.63±0.14** |
| Unknown | 1.6±0.4 | 1.8±0.4 | **1.2±0.2** | 1.8±0.6 | 1.7±0.5 | 1.7±0.3 | 2.1±0.5 | 1.4±0.4 | **1.3±0.3** |
| Number of increased compounds compared to Px | | 21 | 11 | | 34* | 24 | | 21 | 11 |
| Number of decreased compounds compared to Px | | 30 | 40*** | | 17 | 27 | | 30 | 40*** |

B)

| Compound | | OH | | | KIM | | | WIN | |
|---|---|---|---|---|---|---|---|---|---|
| | Mb | Bb(7d)+Mb | Bb(14d)+Mb | Mb | Bb(7d)+Mb | Bb(14d)+Mb | Mb | Bb(7d)+Mb | Bb(14d)+Mb |
| **Alcohols** | | | | | | | | | |
| (*Z*)-3-Hexen-1-ol | 34±6.2 | **17.2±2.8** | **18.9±4.6** | 56±14 | **17.6±5.7** | **16.6±4.0** | 12.4±3.5 | 8.3±1.9 | 9.3±4.7 |
| 1-Octen-3-ol | 7.6±1.3 | **12.4±2.2** | **20.3±4.9** | 5.4±1.6 | **11.1±1.9** | **17.7±2.7** | 9.2±1.9 | 10.3±2.8 | 10.4±1.8 |
| **Esters** | | | | | | | | | |
| (*Z*)-2-Penten-1-ol, acetate | **6.2±1.5** | **5.1±0.8** | **2.4±0.3** | 33±13 | **3.6±0.9** | **3.1±0.7** | 2.4±0.5 | 1.9±0.4 | **1.1±0.3** |
| (*Z*)-3-Hexen-1-ol, acetate | 1832±400 | **1179±195** | **869±165** | 3598±811 | **724±158** | **647±156** | 631±140 | **353±66** | **280±120** |
| Hexyl acetate | 9.1±2.3 | **5.1±1.0** | **6.6±1.5** | 33±13 | **3.4±0.8** | **6.0±3.0** | 3.1±0.9 | 2.0±0.5 | **1.4±0.6** |
| (*Z*)-3-Hexenyl butyrate | 1.9±0.5 | 1.3±0.4 | 1.0±0.3 | 7.7±3.2 | **1.4±0.7** | **0.8±0.3** | 2.4±0.7 | **0.9±0.3** | 1.7±1.1 |
| Methyl salicylate | 29±19 | **8.8±1.5** | 11.5±7.6 | 1.6±0.6 | **8.7±4.4** | 1.9±0.8 | 3.7±1.4 | 4.8±2.6 | **24±12** |
| (*Z*)-3-Hexen-1-ol, 2-methylbutanoate | 3.1±0.8 | 2.4±0.6 | **2.0±0.3** | 11.0±3.8 | **0.9±0.2** | **1.2±0.3** | 1.3±0.4 | **0.68±0.13** | **0.9±0.4** |
| (*Z*)-3-Hexen-1-ol, 3-methylbutanoate | 1.8±0.5 | 1.6±0.3 | 1.1±0.4 | 4.0±1.3 | **0.6±0.4** | **0.45±0.16** | 1.2±0.5 | **0.10±0.06** | **0.23±0.17** |
| Linalyl acetate | 2.2±0.6 | **1.1±0.4** | **0.9±0.2** | 1.0±0.2 | **2.7±1.5** | **0.6±0.2** | 1.9±0.7 | **0.44±0.17** | **0.7±0.2** |
| (*Z*)-4-tert-Butylcyclohexyl acetate | 2.8±0.9 | **1.5±0.3** | 1.7±0.4 | 3.2±1.3 | 3.4±1.8 | 1.14±0.16 | 3.0±0.9 | **1.5±0.4** | **1.10±0.13** |
| α-Terpinyl acetate | 0.24±0.06 | **0.19±0.05** | 0.19±0.06 | 0.24±0.07 | 0.35±0.10 | **0.13±0.04** | 0.34±0.11 | 0.23±0.13 | **0.10±0.02** |
| **Ketones** | | | | | | | | | |
| 3-Methyl-2-butanone | 0.8±0.3 | 1.7±0.9 | 0.33±0.14 | 6.9±2.1 | **3.0±0.9** | **1.1±0.3** | 2.6±1.0 | 3.5±1.4 | 3.1±2.2 |
| 3-Pentanone | 2.9±0.4 | 3.3±0.7 | **1.8±0.3** | 8.5±3.6 | **2.8±0.9** | 4.1±2.3 | 4.5±1.2 | 3.5±0.9 | 3.4±1.1 |
| 3-Methyl-2-pentanone | 7.2±3.4 | 7.0±3.1 | 1.7±0.5 | 30±17 | 6.9±3.1 | 1.5±0.3 | 10.8±4.2 | 6.6±1.8 | 9.3±5.4 |
| α-Isomethylionone | 0.8±0.3 | 1.0±0.8 | 0.42±0.08 | 0.9±0.4 | 1.1±0.4 | 0.6±0.3 | 0.61±0.19 | 0.7±0.3 | 0.9±0.6 |
| **S- & N- containing compounds** | | | | | | | | | |
| 3-Methylbutanenitrile | 4.9±1.6 | 4.1±1.3 | 4.3±1.8 | 4.3±2.3 | **0.84±0.16** | **0.44±0.17** | 0.14±0.14 | 0.22±0.13 | 0.06±0.06 |
| Dimethyl disulfide | 4.4±1.3 | 6.6±1.7 | **12.8±3.5** | 9.3±2.2 | 14±6.5 | 15±3.6 | 5.7±0.8 | **8.2±2.1** | **13.9±3.1** |
| Dimethyl trisulfide | 1.8±0.3 | 2.2±0.5 | **3.8±1.4** | 4.2±1.3 | 4.0±1.9 | 3.8±0.8 | 3.0±0.6 | 2.7±0.4 | 2.9±0.6 |
| 3-Butenyl isothiocyanate | 21.7±3.3 | 41±17 | 86±60 | 10.1±3.9 | 24±15 | 19±11 | 123±51 | 83±36 | 140±39 |
| Benzyl nitrile | 38±15 | 28.8±9.0 | 32±11 | 1.7±1.2 | 1.0±0.4 | 2.1±1.5 | 1.30±0.17 | **2.8±0.9** | **1.8±0.8** |
| Indole | 13.0±6.8 | 12.3±2.8 | 7.6±3.6 | 5.9±2.0 | 6.2±2.4 | 3.1±0.9 | 16±11 | 7.0±3.1 | 9.5±4.8 |
| **Terpenoids** | | | | | | | | | |
| α-Thujene | 300±70 | 314±58 | 290±71 | 155±58 | **97±56** | **87±34** | 448±60 | **304±85** | 400±100 |
| α-Pinene | 87±19 | 91±16 | 84±21 | 54±15 | **34±17** | **31.5±7.9** | 125±14 | **83±19** | 103±24 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Sabinene | 681±137 | 813±144 | 733±163 | 433±160 | **260±149** | **232±86** | 1179±157 | **681±189** | 1001±244 |
| β-Pinene | 57±13 | 73±13 | 60±15 | 39±10 | 28±14 | **20.8±6.3** | 99±15 | **64±17** | 76±17 |
| β-Myrcene | 219±50 | 236±43 | 215±50 | 126±46 | **86±42** | **68±25** | 336±46 | **220±62** | 311±80 |
| α-Terpinene | 42±23 | **23.6±5.2** | **21.2±6.3** | 11.5±4.2 | **8.8±5.1** | **6.5±2.4** | 30.8±5.4 | 30.4±7.7 | 28.0±7.6 |
| Limonene | 310±73 | 332±60 | 313±77 | 224±77 | **142±74** | **121±42** | 463±51 | **321±77** | 464±119 |
| 1,8-Cineole | 79±19 | 80±15 | 77±20 | 37±13 | **26±15** | **20.8±7.7** | 84±11 | 67±21 | 79±23 |
| (*E*)-β-Ocimene | 6.9±1.6 | 7.4±1.4 | 6.5±1.7 | 4.1±1.3 | **2.8±1.5** | **2.1±0.9** | 9.6±1.6 | **6.2±1.5** | 8.7±2.4 |
| γ-Terpinene | 28±15 | **16.3±3.5** | **14.9±4.4** | 7.9±2.8 | 6.2±3.5 | **4.7±1.7** | 21.5±3.7 | 20.8±5.1 | 19.7±5.2 |
| (*E*)-β-Terpineol | 2.7±0.6 | 3.1±0.5 | 3.2±0.9 | 1.8±0.7 | **1.0±0.6** | **0.9±0.4** | 4.3±0.6 | **2.2±0.7** | 3.7±1.0 |
| α-Terpinolene | 17.6±8.0 | **11.5±2.5** | **10.4±3.0** | 5.7±2.0 | **4.3±2.4** | **3.2±1.1** | 15.5±2.4 | 13.4±3.1 | 13.6±3.5 |
| Linalool | 2.2±0.3 | 2.3±0.5 | 2.6±0.6 | 1.8±0.7 | 1.0±0.4 | 0.7±0.3 | 4.1±0.7 | **2.4±0.9** | 4.1±1.2 |
| [3](*E*)-DMNT | 30.9±9.4 | **92±53** | **7.9±2.4** | 210±180 | 19±9.6 | 15.6±3.9 | 2.8±0.9 | 1.9±0.6 | **17±13** |
| Menthol | 1.0±0.6 | 0.46±0.15 | 0.6±0.2 | 0.58±0.12 | **7.0±6.0** | 0.46±0.16 | 0.47±0.16 | 0.9±0.6 | 0.44±0.14 |
| 4-Terpineol | 0.64±0.16 | 0.77±0.13 | 0.67±0.16 | 0.39±0.14 | 0.23±0.16 | **0.29±0.10** | 0.87±0.11 | **0.63±0.14** | **1.2±0.3** |
| α-Terpineol | 2.9±0.7 | 6.2±2.4 | 2.8±1.2 | 3.3±2.1 | 1.5±1.0 | 1.2±0.6 | 9.1±3.1 | 4.6±2.4 | 5.2±2.3 |
| β-Cubebene | 0.07±0.03 | **0.20±0.07** | 0.14±0.03 | 0.12±0.04 | 0.10±0.04 | 0.08±0.03 | 0.17±0.05 | **0.06±0.03** | **0.12±0.05** |
| β-Elemene | 17.2±7.9 | 7.1±7.0 | **0.8±0.8** | 5.6±3.2 | 2.9±2.4 | 4.4±2.2 | 1.1±0.7 | **4.9±2.5** | 0.4±0.3 |
| Bicyclosesquiphellandrene | 0.17±0.05 | **0.48±0.09** | 0.31±0.09 | 0.24±0.16 | 0.18±0.06 | 0.25±0.04 | 0.07±0.04 | 0.07±0.03 | **0.4±0.13** |
| (*Z*,*E*)-α-Farnesene | 1.7±0.6 | 2.1±0.5 | 1.0±0.4 | 1.0±0.5 | 0.7±0.4 | 0.46±0.13 | 0.9±0.4 | 0.49±0.14 | 1.2±0.6 |
| β-Chamigrene | 2.4±1.1 | **1.0±0.9** | **0.18±0.06** | 0.7±0.3 | 0.36±0.17 | 0.5±0.2 | 0.39±0.13 | **1.2±0.6** | **0.23±0.06** |
| (*E*,*E*)-α-Farnesene | 36±16 | 54±21 | 28±11 | 28±19 | 20±12 | 8.0±3.2 | 21±11 | 18.5±5.7 | **40±22** |
| Germacrene A | 2.4±1.1 | 1.2±1.1 | **0.17±0.12** | 0.6±0.3 | 0.5±0.4 | 0.6±0.3 | 0.10±0.04 | **0.7±0.4** | 0.07±0.03 |
| (*Z*)-α-Bisabolene | 0.8±0.4 | 0.4±0.4 | **0.04±0.04** | 0.25±0.16 | 0.14±0.12 | 0.19±0.09 | 0.08±0.05 | **0.27±0.12** | 0.02±0.01 |
| Nerolidol | 0.46±0.19 | **0.64±0.18** | 0.65±0.12 | 1.4±0.3 | 1.6±0.7 | **0.7±0.3** | 0.5±0.2 | **0.16±0.07** | 0.37±0.17 |
| **other or unknown** | | | | | | | | | |
| 4-Ethenyl cyclohexene | 2.9±1.4 | **1.3±0.4** | 3.2±2.6 | 2.5±1.5 | 1.6±0.6 | 1.1±0.4 | 1.9±0.9 | 2.2±1.0 | **1.1±0.5** |
| Unknown | 0.8±0.2 | **0.8±0.2** | 0.7±0.2 | 0.9±0.2 | 0.61±0.13 | 0.64±0.16 | 0.9±0.3 | 0.7±0.2 | 0.7±0.2 |
| Unknown | 2.0±0.5 | **1.7±0.5** | **1.4±0.3** | 2.0±0.4 | 1.3±0.2 | **1.3±0.3** | 2.4±0.6 | **1.4±0.4** | **1.5±0.4** |
| Number of increased compounds compared to Mb | | 25 | 14 | | 12 | 7 | | 13 | 13 |
| Number of decreased compounds compared to Mb | | 26 | 37** | | 39*** | 44*** | | 38*** | 38*** |

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

* 0.01< $P \leq 0.05$, ** 0.001< $P \leq 0.01$, *** $P \leq 0.001$) indicate whether the number of volatiles of which the emission increased relative to the emission by plants infested with host alone is significantly different from the number of volatiles of which the emission decreased ($\chi^2$-test).

## Discussion

Our data show that the response of parasitoids to volatiles emitted by plants dually infested with hosts and aphids differed from the response to volatiles emitted by plants infested with hosts alone. After 7 days of aphid feeding both parasitoid species were more attracted to HIPV emitted by plants with dually infested with hosts and aphids than to those emitted by plants damaged by hosts alone. When aphid feeding is extended to 14 days, this is still the case for *M. mediator,* but *D. semiclausum* now preferred volatiles from plants with only hosts over those from plants with hosts and aphids. Plant population did not affect these behavioural choices. In response to dual infestation with *M. brassicae* caterpillars and aphids, significantly more compounds exhibited a reduction than an increase in emission levels compared to levels measured in plant infested with caterpillars alone. This was the case for all three plant populations and the two aphid infestation periods. However, for *P. xylostella* this pattern was only recorded in OH and WIN plants when aphid infestation was extended. Moreover, in KIM plants more compounds were produced at higher than at lower levels when infested with caterpillars (*P. xylostella*) and aphids for 7 days.

Initial aphid infestation for 6 days before the hosts were introduced resulted in enhanced attraction of *D. semiclausum* wasps when the alternative odour source was a plant infested with hosts alone. When aphids infested the plants for 14 days, HIPV preference of *D. semiclausum* was reversed. Extended aphid infestation resulted in a higher aphid density, and this may affect plant volatile biosynthesis and foraging behaviour of parasitoids. The behavioural choices of parasitoids towards HIPV have long been studied under conditions where only a single herbivore species is feeding on a plant. Thus, co-infestation by non-host aphids may enhance or attenuate the attraction of *D. semiclausum* depending on the length of the aphid infestation period and possibly aphid density. Other studies have investigated the effect of simultaneous feeding by host and non-host herbivores on HIPV induction and the response of natural enemies (Dicke et al., 2009; Zhang et al., 2009; Erb et al., 2010; Zhang et al., 2013), but only few of these studies have addressed the effect of non-host infestation period. For instance, infestation with non-prey whiteflies and prey spider mites interfered with the attraction of carnivorous mites that feed on the spider mites (Zhang et al. 2009). The degree of interference increased with whitefly density (Zhang et al. 2009). However, at low whitefly densities some positive effects were recorded for the effect of whiteflies on the attraction of carnivorous mites (Zhang et al. 2009). *Microplitis mediator* was consistently more attracted to plants dually infested with aphids and hosts over plants infested with hosts alone, regardless of the aphid infestation period. The contrasting preferences of *D. semiclausum* and *M. mediator* at 14 days of aphid infestation suggest that the effect of aphid-density or aphid-infestation duration on volatile mediated foraging is parasitoid species specific.

To maximize reproductive success and extend longevity, parasitic wasps need resources, mainly sugar rich resources such as floral nectar (Amat et al., 2012; Harvey et al., 2012). Aphid honeydew can serve as a food source for the parasitoids when nectar is scarce (Faria et al., 2008). *Aphytis melinus* parasitoids commonly feed on honeydew from non-host hemipteran species in the field and more than 50% of the field-collected female parasitoid of this species had fed on honeydew (Tena et al., 2013). The availability of an alternative food source, such as aphid honeydew on dually-infested plants could increase the plant's value to parasitoids (Stapel et al., 1997), which may also explain why wasps preferred the HIPV from plants infested with host caterpillars plus non-host aphids. If products of the aphids themselves enhance the attractiveness of the volatile blend emitted by plants infested with host and aphids, this seems to affect the behaviour of *M. mediator* more consistently than that of *D. semiclausum*. The response of parasitoids to food-indicating stimuli, such as flower colour and odour depends on the hunger state of the individual. Food-deprived wasps prefer odours related to food over odours related to hosts (Wäckers, 1994). In our study, the wasps were provided with fresh honey and sugar water daily, but their hunger status was not tested before the experiments. To what extent aphid honeydew may play a role in the attractiveness of dually-infested plants to wasps remains to be determined.

Host-selection behaviour by parasitoids attacking herbivorous hosts consists of different phases of which habitat and host pant location are considered the first steps and these are often mediated by HIPV (Vet and Dicke, 1992; Vinson, 1998). Here, we showed that aphid feeding on host-infested plants may benefit parasitoids by increased detectability of the HIPV-blend compared to the blend emitted by plants damaged by caterpillars only. The efficiency of a parasitoid to find hosts can be compromised when other herbivore species are feeding on the same plant (De Rijk et al., 2013). Once a host-infested plant has been found, parasitism success depends on the ability to find the hosts on that plant, to parasitize the hosts and for the parasitoid offspring to successfully develop inside or on these hosts (Vet and Dicke, 1992; Vinson, 1998). A previous study on host quality mediated by the host plant using the same tritrophic systems as in this study reported that development of *P. xylostella* and *D. semiclausum*, but not that of *M. brassicae* and *M. mediator* was positively affected by aphid presence (Li et al., 2014). Body mass of *P. xylostella* and, thus, the amount of biomass available for its parasitoids, was positively affected by a low density of co-infesting *B. brassicae* aphids and negatively by a high density of co-infested aphids (Kroes et al., 2015). These results suggest that the direction of aphid-induced effects on both the performance and behaviour of parasitoids can be aphid density depended. To determine whether successful location of host-infested plants increases successful parasitism and ultimately leads to higher reproductive success needs to be assessed for the two parasitoids investigated here considering the other steps that ultimately determine parasitism success (see e.g. Soler et al., 2007; Wei et al., 2011).

The effect of aphid infestation on the emission rates of plant volatiles was mostly negative, although higher emission rates from plants dually infested with aphids and hosts did occur as well. The negative effects of aphid infestation on volatile emission rates were most pronounced when plants were infested with *M. brassicae* and aphids irrespective of the duration of the aphid infestation, which may result from JA-SA antagonism. In plants infested with *P. xylostella* reduced emission rates were only recorded in OH and WIN plants after extended aphid feeding. Interestingly, plants infested with *M. brassicae* caterpillars plus aphids produced many compounds in lower concentrations than plant infested with hosts alone, but were more attractive to *M. mediator*. This suggests that higher concentrations of HIPV do not necessarily result in increased attraction, which has been reported for other biotic interactions as well. For example, root colonization with non-pathogenic rhizobacteria modified the composition of the volatile blend induced by aphids; most of the compounds were emitted in larger amounts by plants co-infested with root-colonizing rhizobacteria plus aphids than by plants only infested with aphids (Pineda et al., 2013). However, the aphid parasitoid (*Diaeretiella rapae*) preferred the volatile blend from aphid-infested plants without rhizobacteria over the blend from rhizobacteria-treated and aphid-infested plants (Pineda et al., 2013). Linking parasitoid behaviour and chemistry for the *P. xylostella-D. semiclausum* interaction is even more difficult because, here, there were also plant-population specific responses in terms of volatile emission rates (OH and WIN vs KIM). Aphid interference with HIPV induction was shown in OH and WIN when aphid infestation was extended and also negatively affected *D. semiclausum* supporting a role for JA-SA antagonism both behaviourally and chemically.

Enhanced attraction to *P. xylostella*-damaged plants co-infested with aphids for 7 days could not be explained by the data on headspace volatiles. These results suggest that differences in the behavioural response of parasitoids can be caused by very subtle differences in the HIPV blends that are beyond the detection limit of the analytical equipment. Moreover, the two parasitoid species clearly differ in the specific blend characteristics that they use during foraging (Vet and Dicke 1992, Steidle and van Loon 2003). This may result from differences in the sensitivity to specific compounds, the range of compounds that triggers a sensory response, and the processing of stimuli in the brain and is likely to be determined by the ecology and genetic constraints of the parasitoid species.

Genotypes and plants from different populations of the same species can vary in HIPV blends emitted in response to an individual herbivore species (Lou et al., 2006; Casey M. Delphia et al., 2009; Gols et al., 2009). A previous study has demonstrated that plants from the three wild cabbage populations vary in their HIPV profiles in response to infestation by *Pieris rapae* caterpillars (Gols et al., 2011). Here, we investigated dual infestation and recorded that the preference patterns of the parasitoids for volatiles from host-infested plants and plants infested by hosts and non-host aphids were similar across the three wild cabbage populations.

In nature, plants are rarely attacked by one herbivore species and parasitoids have evolved to forage in complex environments. More and more studies have started to investigate foraging behaviour of parasitoids in a multi-herbivore context, albeit often with single non-host densities. Understanding how parasitoids are affected by different non-host densities will be a first step to further unravel how parasitoids forage in dynamic assemblages of herbivore-plant-associations. Enhancing such knowledge will be important to improve pest control strategies that are based on biological control services provided by parasitoids.

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## References

Agbogba BC, Powell W (2007) Effect of the presence of a nonhost herbivore on the response of the aphid parasitoid *Diaeretiella rapae* to host-infested cabbage plants. Journal of Chemical Ecology 33: 2229-2235.

Amat I, Besnard S, Foray V, Pelosse P, Bernstein C, Desouhant E (2012) Fuelling flight in a parasitic wasp: which energetic substrate to use? Ecological Entomology 37: 480-489

Beckers GJM, Spoel SH (2006) Fine-tuning plant defence signalling: salicylate versus jasmonate. Plant Biology 8: 1-10.

Bonaventure G, VanDoorn A, Baldwin IT (2011) Herbivore-associated elicitors: FAC signaling and metabolism. Trends in Plant Science 16: 294-299.

Bylesjö M, Rantalainen M, Cloarec O, Nicholson JK, Holmes E, Trygg J (2006) OPLS discriminant analysis: combining the strengths of PLS-DA and SIMCA classification. Journal of Chemometrics 20: 341-351.

Casey M. Delphia, Jason R. Rohr, Andrew G. Stephenson, Moraes Consuelo MD, Mark C. Mescher (2009) Effects of genetic variation and inbreeding on volatile production in a field population of Horsenettle. International Journal of Plant Sciences 170: 12-20.

De Moraes C, Lewis W, Pare P, Alborn H, Tumlinson J (1998) Herbivore-infested plants selectively attract parasitoids. Nature 393: 570-573.

De Rijk M, Dicke M, Poelman EH (2013) Foraging behaviour by parasitoids in multiherbivore communities. Animal Behaviour 85: 1517-1528.

Delphia CM, Mescher MC, De Moraes CM (2007) Induction of plant volatiles by herbivores with different feeding habits and the effects of induced defenses on host-plant selection by thrips. Journal of Chemical Ecology 33: 997-1012.

Dicke M (2009) Behavioural and community ecology of plants that cry for help. Plant, Cell and Environment 32: 654-665.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends in Plant Science 15: 167-175.

Dicke M, Van Loon JJA, Soler R (2009) Chemical complexity of volatiles from plants induced by multiple attack. Nature Chemical Biology 5: 317-324.

Dicke M, van Poecke RM, de Boer JG (2003) Inducible indirect defence of plants: from mechanisms to ecological functions. Basic and Applied Ecology 4: 27-42.

Engelberth J, Koch T, Schüler G, Bachmann N, Rechtenbach J, Boland W (2001) Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. Plant Physiology 125: 369-377.

Erb M, Foresti N, Turlings TCJ (2010) A tritrophic signal that attracts parasitoids to host-damaged plants withstands disruption by non-host herbivores. BMC Plant Biology 10: 247.

Eriksson L, Byrne T, Johansson E, Trygg J, Vikström C (2013) Multi-and megavariate data analysis: basic principles and applications, Ed Third revised. Umetrics Academy, Malmö, Swenden.

Faria CA, Wäckers FL, Turlings TCJ (2008) The nutritional value of aphid honeydew for non-aphid parasitoids. Basic and Applied Ecology 9: 286-297.

Gols R, Bullock JM, Dicke M, Bukovinszky T, Harvey JA (2011) Smelling the wood from the trees: non-linear parasitoid responses to volatile attractants produced by wild and cultivated cabbage. Journal of Chemical Ecology 37: 795-807.

Gols R, van Dam NM, Raaijmakers CE, Dicke M, Harvey JA (2009) Are population differences in plant quality reflected in the preference and performance of two endoparasitoid wasps? Oikos 118: 733-743.

Gols R, Wagenaar R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM, Harvey JA (2008) Genetic variation in defense chemistry in wild cabbages affects herbivores and their endoparasitoids. Ecology 89: 1616-1626.

Guerrieri E, Digilio MC (2008) Aphid-plant interactions: a review. Journal of Plant Interactions 3: 223-232.

Hare JD (2011) Ecological role of volatiles produced by plants in response to damage by herbivorous insects. Annual Review of Entomology 56: 161-180.

Harvey JA, Cloutier J, Visser B, Ellers J, Wäckers FL, Gols R (2012) The effect of different dietary sugars and honey on longevity and fecundity in two hyperparasitoid wasps. Journal of Insect Physiology 58: 816-823.

Heil M (2008) Indirect defence via tritrophic interactions. New Phytologist 178: 41-61.

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annual Review of Plant Biology 59: 41-66.

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science 291: 2141-2144.

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annual Review of Plant Biology 53: 299-328.

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiology 146: 839-844.

Kroes A, van Loon JJA, Dicke M (2015) Density-dependent interference of aphids with caterpillar-induced defenses in Arabidopsis: involvement of phytohormones and transcription factors. Plant and Cell Physiology 0: 1-9.

Kunert M, Biedermann A, Koch T, Boland W (2002) Ultrafast sampling and analysis of plant volatiles by a hand-held miniaturised GC with pre-concentration unit: kinetic and quantitative aspects of plant volatile production. Journal of Separation Science 25: 677-684.

Li Y, Dicke M, Harvey J, Gols R (2014) Intra-specific variation in wild *Brassica oleracea* for aphid-induced plant responses and consequences for caterpillar–parasitoid interactions. Oecologia 174: 853-862.

Lou Y, Hua X, Turlings TJ, Cheng J, Chen X, Ye G (2006) Differences in induced volatile emissions among rice varieties result in differential attraction and parasitism of *Nilaparvata lugens* eggs by the parasitoid *Anagrus nilaparvatae* in the field. Journal of Chemical Ecology 32: 2375-2387.

Meiners T (2015) Chemical ecology and evolution of plant–insect interactions: a multitrophic perspective. Current Opinion in Insect Science 8: 22-28.

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in *Arabidopsis* in relation to plant defense pathways. Plant Physiology 125: 1074-1085.

Mumm R, Dicke M (2010) Variation in natural plant products and the attraction of bodyguards involved in indirect plant defense. Canadian Journal of Zoology 88: 628-667.

Ozawa R, Arimura G, Takabayashi J, Shimoda T, Nishioka T (2000) Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. Plant and Cell Physiology 41: 391-398.

Pineda ANA, Soler R, Weldegergis BT, Shimwela MM, Van Loon JJA, Dicke M (2013) Non-pathogenic rhizobacteria interfere with the attraction of parasitoids to aphid-induced plant volatiles via jasmonic acid signalling. Plant, Cell and Environment 36: 393-404.

Poelman EH, Broekgaarden C, Van Loon JJA, 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 EH, Bruinsma M, Zhu F, Weldegergis BT, Boursault AE, Jongema Y, van Loon JJA, Vet LE, Harvey JA, Dicke M (2012) Hyperparasitoids use herbivore-induced plant volatiles to locate their parasitoid host. PLoS Biology 10: e1001435.

Poelman EH, Harvey JA, van Loon JJA, Vet LEM, Dicke M (2013) Variation in herbivore-induced plant volatiles corresponds with spatial heterogeneity in the level of parasitoid competition and parasitoid exposure to hyperparasitism. Functional Ecology 27: 1107-1116.

Rodriguez-Saona C, Chalmers JA, Raj S, Thaler JS (2005) Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. Oecologia 143: 566-577.

Rodriguez-Saona C, Crafts-Brandner S, Cañas L (2003) Volatile emissions triggered by multiple herbivore damage: beet armyworm and whitefly feeding on cotton plants. Journal of Chemical Ecology 29: 2539-2550.

Schmelz EA, Engelberth J, Alborn HT, O'Donnell P, Sammons M, Toshima H, Tumlinson JH (2003) Simultaneous analysis of phytohormones, phytotoxins, and volatile organic compounds in plants. Proceedings of the National Academy of Sciences 100: 10552-10557.

Schoonhoven LM, van Loon JJA, Dicke M (2005) Insect-Plant Biology, 2nd edn. Oxford University Press, Oxford, UK.

Soler R, Badenes-Perez FR, Broekgaarden C, Zheng SJ, David A, Boland W, Dicke M (2012) Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. Functional Ecology 26: 156-166.

Soler R, Harvey JA, Kamp AFD, Vet LEM, Van der Putten WH, Van Dam NM, Stuefer JF, Gols R, Hordijk CA, Bezemer TM (2007) Root herbivores influence the behaviour of an aboveground parasitoid through changes in plant-volatile signals. Oikos 116: 367-376.

Stapel JO, Cortesero AM, De Moraes CM, Tumlinson JH, Lewis WJ (1997) Extrafloral nectar, honeydew, and sucrose effects on searching behavior and efficiency of *Microplitis croceipes* (Hymenoptera: Braconidae) in cotton. Environmental Entomology 26: 617-623.

Steinberg S, Dicke M, Vet L, Wanningen R (1992) Response of the braconid parasitoid *Cotesia* (= *Apanteles*) *glomerata* to volatile infochemicals: effects of bioassay set-up, parasitoid age and experience and barometric flux. Entomologia experimentalis et applicata 63: 163-175.

Takabayashi J, Sabelis MW, Janssen A, Shiojiri K, van Wijk M (2006) Can plants betray the presence of multiple herbivore species to predators and parasitoids? The role of learning in phytochemical information networks. Ecological Research 21: 3-8.

Tena A, Pekas A, Wackers FL, Urbaneja A (2013) Energy reserves of parasitoids depend on honeydew from non-hosts. Ecological Entomology 38: 278-289.

Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science 17: 260-270.

Turlings TC, Bernasconi M, Bertossa R, Bigler F, Caloz G, Dorn S (1998) The induction of volatile emissions in maize by three herbivore species with different feeding habits: possible consequences for their natural enemies. Biological Control 11: 122-129.

Turlings TCJ, Tumlinson JH, Lewis WJ (1990) Exploitation of herbivore-induced plant odors by host-seeking parasitic wasps. Science 250: 1251-1253.

Vet LEM, Dicke M (1992) Ecology of infochemical use by natural enemies in a tritrophic context. Annu Rev Entomol 37: 141-172.

Vinson SB (1998) The general host selection behavior of parasitoid Hymenoptera and a comparison of initial strategies utilized by larvaphagous and oophagous species. Biological Control 11: 79-96.

Wäckers F (1994) The effect of food deprivation on the innate visual and olfactory preferences in the parasitoid *Cotesia rubecula*. Journal of Insect Physiology 40: 641-649.

Wei JN, Wang LH, Zhao JH, Li CY, Ge F, Kang L (2011) Ecological trade-offs between jasmonic acid-dependent direct and indirect plant defences in tritrophic interactions. New Phytologist 189: 557-567.

Zarate SI, Kempema LA, Walling LL (2007) Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiology 143: 866-875.

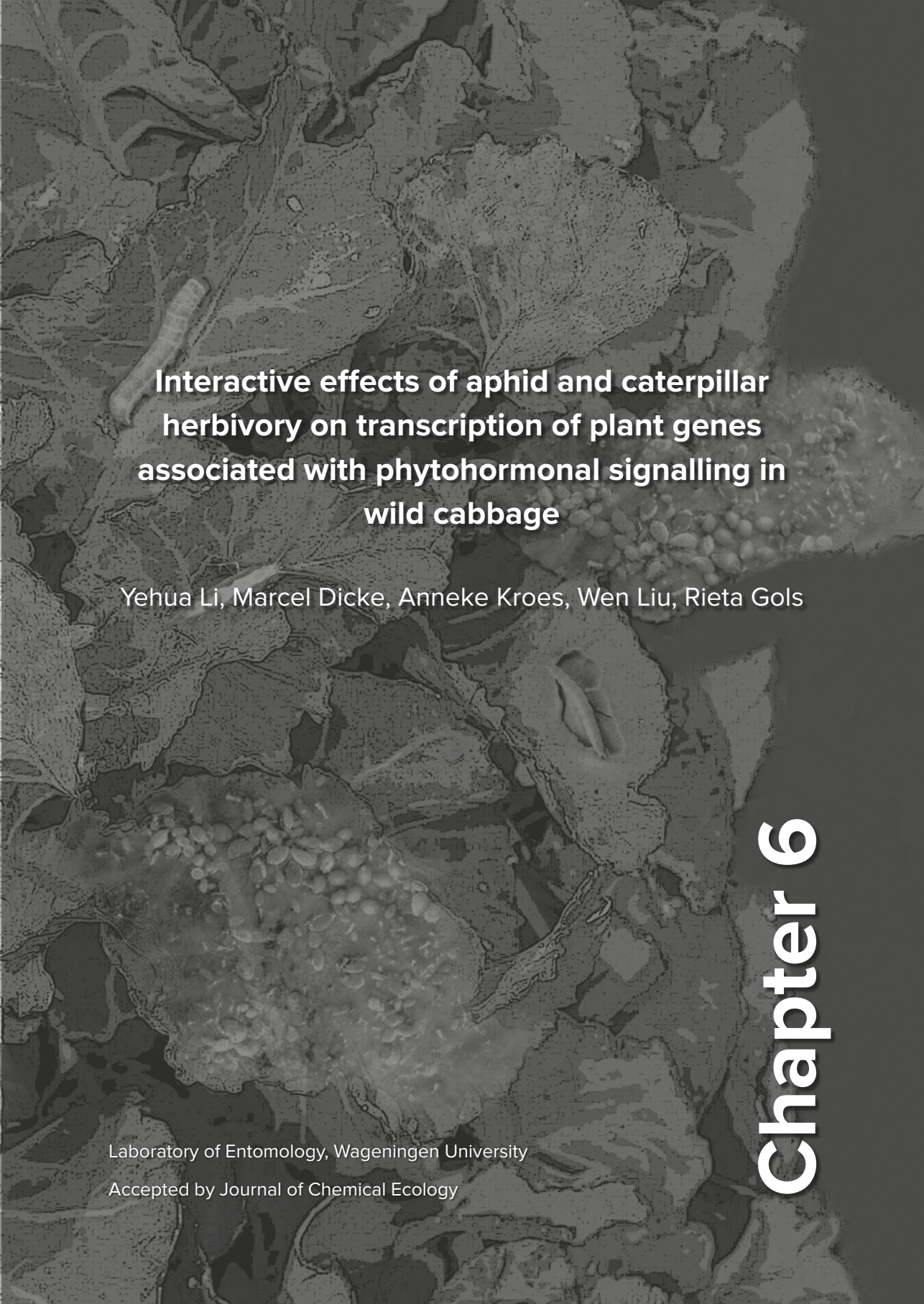
Zhang P, Broekgaarden C, Zheng S, Snoeren TAL, Loon JJAv, Gols R, Dicke M (2013) Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. New Phytologist 197: 1291-1299.

Zhang PJ, Zheng SJ, van Loon JJA, Boland W, David A, Mumm R, Dicke M (2009) Whiteflies interfere with indirect plant defense against spider mites in Lima bean. Proceedings of the National Academy of Sciences of the United States of America 106: 21202-21207.

## Appendix

**Appendix 1** Summary of significance levels and parameters of OPLS-DA models comparing volatile emissions from plants with single and dual herbivore infestations. Each cabbage population (*Brassica oleracea*: OH, KIM, WIN) was singly infested with 10 L2 *Plutella xylostella* or 30 L1 *Mamestra brassicae* caterpillars for 24 hours, or dually infested with both 20 adult aphids (*Brevicoryne brassicae*) for 7 / 14 days and either caterpillar species for 24 h.

| Treatment comparisons between single and dual infestation | Cabbage population | Significant model? | Model type | PCs | R2X(cum) | R2Y(cum) | Q2(cum) |
|---|---|---|---|---|---|---|---|
| *P. xylostella* vs aphids (7d) + *P. xylostella* | OH | No | OPLS-DA | 1+1+0 | 0.278 | 0.72 | -0.165 |
| | KIM | No | OPLS-DA | 0+0+0 | | | |
| | WIN | No | OPLS-DA | 0+0+0 | | | |
| *P. xylostella* vs aphids (14d) + *P. xylostella* | OH | No | OPLS-DA | 1+1+0 | 0.33 | 0.806 | -0.109 |
| | KIM | Yes | OPLS-DA | 1+1+0 | 0.309 | 0.899 | 0.501 |
| | WIN | No | OPLS-DA | 1+1+0 | 0.468 | 0.689 | 0.0868 |
| *M. brassicae* vs aphids (7d) + *M. brassicae* | OH | No | OPLS-DA | 1+1+0 | 0.371 | 0.819 | 0.19 |
| | KIM | No | OPLS-DA | 1+1+0 | 0.457 | 0.524 | 0.0975 |
| | WIN | No | OPLS-DA | 1+1+0 | 0.437 | 0.65 | -0.143 |
| *M. brassicae* vs aphids (14d) + *M. brassicae* | OH | No | OPLS-DA | 1+1+0 | 0.423 | 0.685 | 0.363 |
| | KIM | No | OPLS-DA | 1+2+0 | 0.529 | 0.888 | 0.471 |
| | WIN | No | OPLS-DA | 1+1+0 | 0.573 | 0.608 | 0.288 |



# Interactive effects of aphid and caterpillar herbivory on transcription of plant genes associated with phytohormonal signalling in wild cabbage

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## Abstract

Plants are commonly attacked by a variety of insect herbivores, and they have developed specific defences against different types of attackers. At the molecular level, herbivore-specific signalling pathways are activated by plants in response to attackers with different feeding strategies. Feeding by leaf-chewing herbivores predominantly activates JA-regulated defences, whereas feeding by phloem-sucking herbivores generally activates SA-regulated defences. When challenged sequentially by both phloem-sucking and leaf-chewing herbivores, SA-JA antagonism may constrain the plant's ability to timely and adequately divert the defence against the second herbivore that requires activation of a different defensive pathway. We investigated the effect of the temporal sequence of infestation by the aphid *Brevicoryne brassicae* and three caterpillar species, *Plutella xylostella*, *Pieris brassicae*, and *Mamestra brassicae,* on the interaction between JA and SA signal-transduction pathways in three wild cabbage populations. We found no support for SA-JA antagonism, irrespective of the temporal sequence of herbivore introduction or the identity of the caterpillar species based on the expression levels of the JA- and SA-regulated marker genes *LOX* and *PR-1,* respectively. In general, infestation with aphids alone had little effect on the transcript levels of the two marker genes, whereas the three caterpillar species upregulated not only *LOX* but also *PR-1*. Transcriptomic changes were different for plants from the three different natural cabbage populations.

**Keywords:** Aphid infestation, caterpillar infestation, gene expression, genotypic variation, plant defence, SA-JA antagonism.

## Introduction

Plants live in a hostile environment and are challenged by a diverse range of attackers, including microbial pathogens and insect herbivores that may attack the plant either simultaneously or sequentially. To cope with the diversity of biotic threats that may reduce survival and fitness of plants, they are equipped with traits that prevent or reduce attack by biotic agents. These traits, both physical and chemical, can be constitutively expressed or may be activated or enhanced upon attack (Agrawal 1999; Dicke and Baldwin 2010; Karban and Baldwin 1997). To respond adequately to biotic threats, plants need to detect and differentiate between different attacker species. Following the perception and recognition of the attacking herbivore, plants activate an herbivore-specific signal-transduction network that leads to biochemical and physiological changes in the plants (De Vos et al. 2005; Erb et al. 2012; Wu and Baldwin 2009; Wu and Baldwin 2010).

Specificity in the response to attackers allows plants to mount a defence that can more effectively cope with herbivore species with distinct life styles and feeding strategies (Howe and Jander 2008; Pieterse et al. 2009). According to their feeding modes, herbivores can be broadly grouped into leaf chewers and phloem feeders. Leaf chewers remove plant tissues and can cause severe damage to the plants, whereas individuals of piercing-sucking phloem-feeding herbivores feed more subtly, causing only minimal damage to other plant tissues (Schoonhoven et al. 2005). At the molecular level, defences against leaf-chewing and phloem-feeding herbivores are regulated by two major signal-transduction pathways controlled by the phytohormones jasmonic acid (JA), and salicylic acid (SA). Although there are exceptions, chewing herbivores generally activate defence responses regulated by JA, whereas piercing-sucking herbivores activate defence responses regulated by SA (Howe and Jander 2008; Kessler and Baldwin 2002; Mewis et al. 2005; Thaler et al. 2012).

When plants are challenged by multiple herbivore species, crosstalk between defence-related phytohormonal signalling pathways may occur, which can help plants to fine-tune their response timely and plastically to the attackers encountered (Howe and Jander 2008; Pieterse et al. 2009; Stam et al. 2014). The best studied interaction between phytohormonal signalling pathways is the antagonistic interaction between JA- and SA-mediated signalling. The activation of the JA signalling pathway may interfere with the SA signalling pathway and *vice versa* when challenged simultaneously by leaf-chewing and phloem-feeding herbivores (Koornneef and Pieterse 2008; Pieterse et al. 2012; Thaler et al. 2012). The ecological consequence of this negative SA-JA crosstalk includes e.g. the enhanced performance of caterpillars and their parasitoids on aphid-infested plants as a result of the interference of SA signalling with JA-induced plant defences (Li et al. 2014; Rodriguez-Saona et al. 2010; Soler et al. 2012). The negative interaction between JA- and SA-mediated signalling suggests that plants may also face trade-offs in their ability to cope with multiple stress factors (Zarate et al. 2007; Zhang et al. 2009).

The temporal sequence of herbivory may determine the outcome of the SA-JA crosstalk (Mouttet et al. 2013). For instance, a study on *Nicotiana attenuata* showed that the order of attack by phloem-feeding mirids and leaf-chewing tobacco hornworms is an important determinant explaining the differences in plant transcriptional responses (Voelckel and Baldwin 2004). Moreover, the leaf chewing *Spodoptera frugiperda* negatively affected the colonization of maize plants by the root feeder *Diabrotica virgifera*, but only when the leaf herbivore arrived earlier than the root herbivore (Erb et al. 2011). Thus, when attacked by different species sequentially, the kinetics of the plant's response to the first attacker may limit the ability of the plant to divert its response to a second attacker that activates a different signal-transduction pathway. Yet, in Lima bean plants the order of attack by JA-activating spider mites and SA-activating whiteflies did not exhibit major effects on induced plant responses (Zhang et al. 2009).

Although herbivores of the same feeding guild generally trigger the same major signalling pathway (Erb et al. 2012), plant responses to species of the same feeding guild are not exactly the same (Bidart-Bouzat and Kliebenstein 2011), which is likely the result of modulation of defence responses due to crosstalk at the molecular level (De Vos et al. 2005). For example, feeding by various lepidopteran species resulted in differential induction of the three major phytohormones involved in induced plant responses, as well as differential transcriptional responses (Diezel et al. 2009; Poelman et al. 2011; Zhu et al. 2015).

Closely related plant species may vary in their responses to the same type of herbivory (Schmidt et al. 2005). Moreover, within one species, heritable variation in resistance traits is an important component in the adaptation of plants to environmental stresses (Gols et al. 2008; Newton et al. 2009a; Wu and Baldwin 2010). Intraspecific variation was found for plant secondary metabolites, such as glucosinolates in brassicaceous plant species, in *Arabidopsis* accessions and wild cabbage, *Brassica oleracea*, populations (Gols et al. 2008; Kliebenstein et al. 2001; Newton et al. 2009b) and crosstalk between SA- and JA-regulated defences differed among *Arabidopsis* accessions (Pieterse et al. 2009; Pieterse and Dicke 2007; Traw et al. 2003). In two accessions of *N. attenuata,* large differences in herbivory-induced early signalling events, such as MAPK activity, JA and ethylene production, and transcript accumulation of genes encoding transcription factors were recorded (Wu et al. 2008). Therefore, the underlying regulatory mechanisms of plant defence may vary among plant genotypes and populations.

The aim of this study was to investigate whether aphid- and caterpillar-induced plant responses interfere with each other through negative SA-JA crosstalk in different populations of wild cabbage. Underlying mechanisms explaining plant responses to herbivory rely to a large extent on studies performed on *A. thaliana* (De Vos et al. 2005; Koornneef and Pieterse 2008; Kroes et al. 2015; Pieterse et al. 2009). The question is to what extent the results of these studies are representative for plant responses to herbivory

in general or for brassicaceous plants more specifically, as the interaction of Arabidopsis with herbivores in nature is limited due to their short life cycle early in the growing season (Harvey et al. 2007).

In this study, we used plants grown from seeds that originate from three wild cabbage populations that are known to differ in secondary plant chemistry (Gols et al. 2008; Harvey et al. 2011; Newton et al. 2009a), and interact in nature with the herbivores used in this study, the aphid *Brevicoryne brassicae* L. (Hemiptera, Aphididae), and three chewing lepidopteran species, caterpillars of *Plutella xylostella* (L.) (Plutellidae), *Pieris brassicae* L. (Pieridae) and *Mamestra brassicae* L. (Noctuidae), respectively (Newton et al. 2009b). We addressed the following questions: 1) what is the effect of the sequence of herbivore attack on SA-JA crosstalk, 2) how general is this response when using different species of chewing herbivores, and 3) is there intra-specific variation in the plant's responses to herbivory by aphids and caterpillars? We quantified the expression levels of two marker genes related to JA- and SA-signalling, i.e. *LIPOXYGENASE* (*LOX*) and *PATHOGENESIS-RELATED PROTEIN-1* (*PR-1*), respectively (Bell et al. 1995; Jirage et al. 2001), at different time points following inoculation by each of the three different chewing herbivore species and the piercing-sucking aphid when introduced alone, simultaneously or sequentially on wild cabbage plants from populations.

## Material and Methods

### Plants and insects

Seeds of wild cabbage (*Brassica oleracea*) populations were collected in Dorset, U.K., at sites known as Kimmeridge (50°36'N, 2°07'W), Old Harry (50°38'N, 1°55'W), and Winspit (50°35'N, 2°02'W), hereafter called KIM, OH and WIN, respectively. Plants were grown from seeds in 1.5-L pots (1 plant per pot) containing potting soil (Lentse potgrond no. 4; Lent, The Netherlands) in a greenhouse (22 ± 3 °C, 50–70 % relative humidity [RH], light:dark regime [L:D] 16:8 h). Plants were placed in large trays (675 ×170 cm) that were automatically flooded with water and nutrients ($NH_4$ 1.2, K 7.2, Ca 4.0, Mg 1.82, $NO_3$ 12.4, $SO_4$ 3.32, P 1.0, Fe 35.0, Mn 8.0, Zn 5.0, B 20.0, Cu 0.5, Mo 0.5 in mmol/L) once every day for 20 min.

Except for *M. brassicae,* all other herbivore species (*B. brassicae*, *P. xylostella*, and *P. brassicae*) are specialist feeders on brassicaceous plant species, although *M. brassicae* is considered a pest on cabbage crops like the other three herbivore species. All insect cultures were maintained on Brussels sprouts (*B. oleracea* L. var gemmifera cv. Cyrus) plants in a greenhouse or a climate room at 22 ± 2˚C, 60-70% RH and 16:8 h L:D photoregime.

**General treatment and sampling protocol**

In a pilot experiment, we first determined the amount of damage inflicted in 24 h by each caterpillar species and then adjusted the number of caterpillars per species to standardize the consumed area of leaf tissues. We used a transparent plastic sheet with 1-mm$^2$ grid to quantify the area of the consumed leaf tissue in mm$^2$. Results of the pilot showed that three two-day-old second instar (L2) *P. xylostella*, four one-day-old L1 *M. brassicae*, and three neonate *P. brassicae* larvae, respectively, consume similar amounts of leaf tissue (*P. xylostella,* 53 ± 4.6; *M. brassicae,* 59 ± 6.0; *P. brassicae,* 49.9 ± 2.3 mm$^2$) in 24 h. These numbers of caterpillars were used to inoculate the plants in the experiments described below. The initial inoculation density of *B. brassicae* was set at 8 adult aphids per plant.

Plants were exposed to herbivory treatments when they were four weeks old. Insects were introduced onto the first fully expanded leaf. To confine the insects to this leaf, the leaf petiole was wrapped with cotton wool. In each of the three experiments described below, one set of plants served as a control and was not exposed to herbivory, but was otherwise treated similarly. Plants of the three cabbage populations, exposed to different herbivory treatments, were placed randomly on the tables in a greenhouse. For gene-expression quantification, two leaf discs were punched with a cork-borer (diameter 1.8 cm) from the herbivore-exposed leaves, immediately after removal of the insects. Leaf discs were collected from three plants and pooled (=one replicate sample). Twelve plants were prepared to obtain four replicate samples in total for each plant population, herbivore treatment and time-point combination (see below). At each time point, an equal number of samples was collected from control plants and a new cohort of control plants was used at each time point. Immediately after sample collection, samples were flash-frozen in liquid nitrogen and stored in a freezer at -80˚C till further processing for qRT-PCR.

***Experiment 1: single versus dual infestation with B. brassicae aphids and P. xylostella caterpillars***

Plants were inoculated with either *B. brassicae* (B) or *P. xylostella* (Px), or a combination of simultaneous *B. brassicae* and *P. xylostella* inoculation (Px+B). Samples for gene expression were collected at 6, 24 and 48h after introduction of the herbivores as described in the previous section (Fig 1A).

***Experiment 2: effect of the order of arrival of B. brassicae aphids and P. xylostella caterpillars***

Plants were initially inoculated with *B. brassicae* aphids or *P. xylostella* caterpillars, or left free of herbivores. The insects were allowed to feed and reproduce (aphids only) for five days. Following this incubation with the first herbivore, half of the plants exposed to each of the two herbivore treatments were co-infested with the other herbivore (coded BPx and

PxB, Fig 1B), whereas the remaining half of the plants were left as they were, (B and Px, Fig 1B). In addition, cohorts of plants that had not been exposed to herbivores previously, were inoculated with either *B. brassicae* or *P. xylostella* caterpillars (CB and CPx, in Fig 1B). Samples for gene expression were collected from all plants including controls (=without any herbivory) at 24 and 48h after the second herbivore had been introduced.

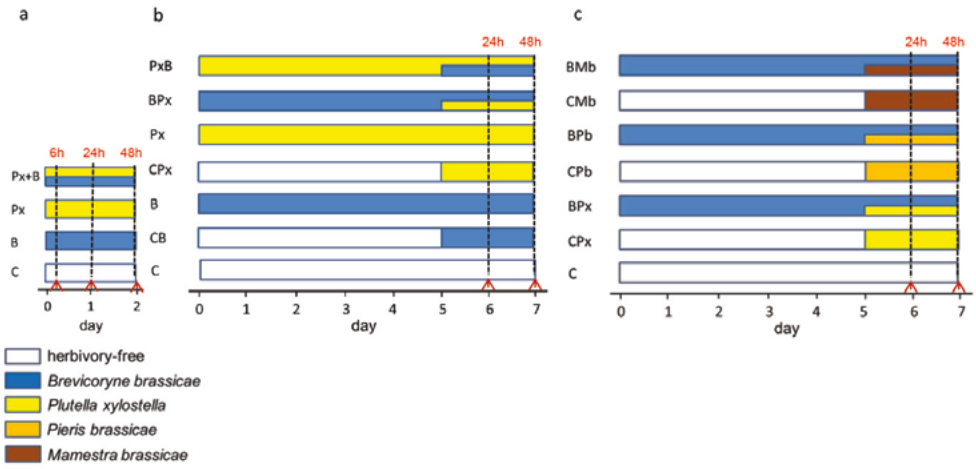


**Figure 1.** The temporal sequence of plant inoculation with different insect herbivore species and their coding in the three experiments (panels a-c). Four-week-old plants were inoculated with the first herbivore species on day 0. In experiment 1, in the dual-infestation treatment, both herbivores were introduced onto the plants simultaneously on day 0. In experiments 2 (panel b) and 3 (panel c), the second herbivore was introduced on day 5. Leaf tissues for gene expression analysis were collected at 6, 24 and 48h following inoculation with the herbivores in experiment 1, and at 24 and 48h following inoculation with the second herbivore in experiments 2 and 3 (sampling time points are indicated by the red coloured arrows on the X-axis).

***Experiment 3: effect of sequential infestation with B. brassicae aphids and caterpillars of different herbivore species***

Sets of plants were or were not inoculated with *B. brassicae* aphids and incubated for five days and were then infested with caterpillars of one of three different lepidopteran species, i.e. *P. xylostella*, *P. brassicae* or *M. brassicae* (without aphids CPx, CPb and CMb, and with aphids BPx, BPb and BMb, Fig 1C). Samples for gene expression were collected from all plants including controls (=without any herbivory) at 24 and 48h after the second herbivore had been introduced.

### RNA isolation and real-time quantitative Reverse Transcription PCR (qRT-PCR)

Samples were kept frozen using liquid nitrogen and ground to a fine powder with a mortar and pestle. RNA was isolated from homogenised material using RNeasy Plant Mini Kit

(Qiagen) and treated with DNAsel (Invitrogen) following the manufacturer's instructions. After isolation, the RNA concentration and purity were measured using a NanoDrop ND-100 (NanoDrop Technologies, Wilmington, DE, USA) spectrophotometer (all samples with OD 260 nm / 280 nm of 1.9–2.2 ratio). RNA integrity number (RIN) of randomly selected samples was confirmed by Bioanalyzer (Agilent 2100) with Agilent RNA 6000 Nano Kit (Agilent Technologies, Waldbronn, Germany). The concentration of RNA obtained from the plant material was adjusted to 1 µg/µl and subsequently reverse-transcribed into cDNA with the iScript cDNA synthesis Kit (Bio-Rad). RNA samples were randomly selected for a negative control cDNA reaction by omitting the reverse transcriptase, to ensure that no samples were contaminated with genomic DNA. qRT-PCR analysis was performed in an Mx3000P™ real-time PCR Detection system (Rotorgene). The qPCR amplification mix consisted of: 12.5 µl of SYBER Green Supermix (Bio-Rad), 5 µl cDNA, 5.5 µl DEPC, 1µl of a gene-specific primer pair (see Table 1, Keygene, Wageningen, The Netherlands) added up to a total volume of 25 µl. The amplification efficiency of primers was determined by generating standard curves using a 10-fold dilution of the randomly selected samples per treatment and per cabbage population. Each dilution was assayed in triplicate. The amplification efficiency was between 90 – 100 % for all primer pairs tested on the three cabbage populations. For each cDNA sample, qPCR amplification reactions were performed in duplicate. The following PCR program was used for all PCR reactions: an initial denaturation step of 3 minutes at 95°C, followed by 40 cycles of 15 s at 95°C, 45 s at 59°C. At the end of each run, a melting curve analysis was performed to verify that only a single gene transcript had been amplified. Relative gene expression level was calculated by normalizing expression levels to the threshold cycle (Ct) values of the reference gene GAPDH using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001).

**Table 1.** Primer sequences used for amplifying *GAPDH*, *PR-1*, and *LOX* genes of *B. oleracea*

| Gene name | Forward primer | Reverse primer |
|---|---|---|
| *BoGAPDH* | AGAGCCGCTTCCTTCAACATCATT | TGGGCACACGGAAGGACATACC |
| *BoLOX* | AAGGCATCGAGCTTCCCAA | TTGCTTTTCAACGGCCACTC |
| *BoPR-1* | GTCAACGAGAAGGCTAACTATAACTACG | TTACACCTTGCTTTGCCACATCC |



### Statistics

The response variables, relative expression levels of *LOX* and *PR-1,* were log-transformed to meet assumptions of normality and homoscedasticity. Data were analysed using General Linear Model analysis of variance in Genstat (17th edition, VSN International, Hemel Hempstead, UK). In experiments 1 and 2, plant population, herbivore treatment and time points were entered as fixed factors in the statistical model. The data of experiment 2 were split into two sets: data of gene expression levels of control plants and those exposed to aphid infestation alone (C, CB and B) were analysed separately, to confirm the effect of

aphid infestation in experiment 1; data of gene expression levels of plants with caterpillar (*P. xylostella*) infestation alone and in combination with aphid feeding (CPx, Px, BPx, and PxB) were analysed to investigate the effect of temporal order of infestation. In experiment 3, we investigated whether expression levels of the two genes were similarly affected by the infestation of different caterpillar species, both in the presence and absence of aphid feeding. In addition to population and time points, caterpillar species and presence / absence of aphids were entered as fixed terms in the statistical model. When terms in the GLM were significant, pairwise differences among factor levels were determined using Tukey-Kramer-corrected LSD tests.

## Results

### Experiment 1: single versus dual infestation with *B. brassicae* aphids and *P. xylostella* caterpillars

#### *LOX expression*

There was a significant effect of herbivore treatment, time point and population on the expression levels of *LOX* (Table 2, Fig 2A-C). Feeding by *P. xylostella* caterpillars alone or in combination with *B. brassicae* aphids (Fig 1A) similarly up-regulated the expression of *LOX* (Px vs. Px+B, $P$=0.32), whereas expression levels of *LOX* were similar in the controls and in plant exposed to *B. brassicae* alone (C vs. B, $P$=0.71). The significant interaction between herbivore treatment and plant population further indicated that *LOX* expression levels were plant-population specific. All populations responded similarly to the various herbivore treatments but the levels of upregulation depended on plant population (Fig 2A-C). *LOX* transcripts were higher in OH plants than in plants of the other two populations (OH vs. KIM and OH vs. WIN, $P$<0.001), whereas they were similar in KIM and WIN plants ($P$=0.93). The relative expression of *LOX* in caterpillar-exposed plants increased with time. However, the temporal dynamics of this gene differed among the populations (Table 2). For example, in KIM plants *LOX* transcripts were only significantly different in samples taken at 6 and at 48 h following herbivore introduction (KIM-6 vs. KIM-48, $P$=0.02), whereas in WIN plants transcript levels differed at both 24 and 48 h from those at 6 h ($P$<0.001 both comparisons). In OH plants the patterns were similar as in KIM plants but they were not statistically significant due to the high levels of variation (6h vs. 48h $P$=0.10).

#### *PR-1 expression*

The expression levels of *PR-1,* a SA-responsive marker gene, were affected by herbivore treatment and plant population, and the effect of time point was population specific

(Table 2, Fig 2D-F). The expression of this gene was only up-regulated in KIM plants (KIM vs. OH, *P*=0.005, KIM vs. WIN, *P*=0.01; WIN vs. OH, *P*=0.97) and the expression levels only increased in response to feeding by *P. xylostella* alone or in combination with aphids (B vs. C, *P*=0.77, Px vs. Px+B, *P*=0.92, all other pair-wise comparisons *P*<0.05). In KIM plants, *PR-1* transcript levels were higher in tissue sampled at 24 h than in those sampled at 6 h following infestation (KIM-6 vs. KIM-24, *P*=0.007; all other within population-time point comparisons *P*>0.05).

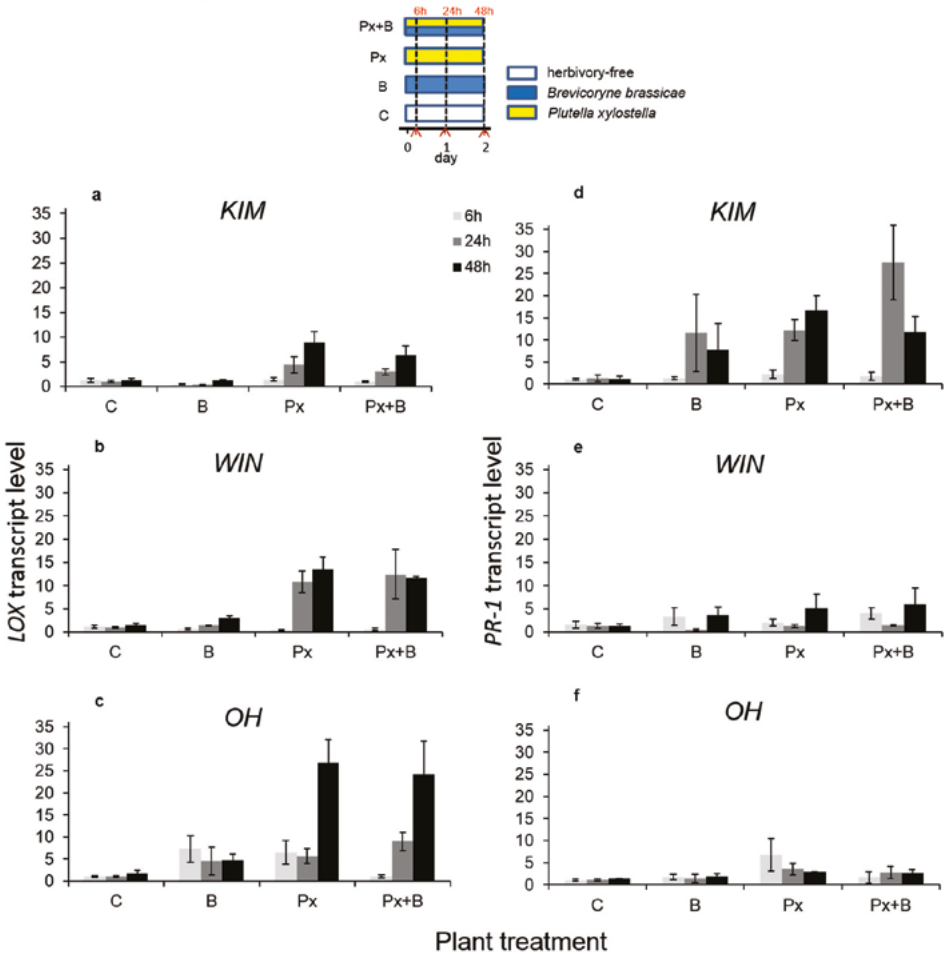


**Figure 2.** qRT-PCR analysis of transcript levels of the JA-responsive defence marker gene *LOX* (panels A-C) and the SA-responsive defence marker gene *PR-1* (panels D-F) in leaves of plants from three different wild *Brassica oleracea* populations (KIM [A;D]; WIN [B;E]; OH [C;F]) at 6, 24, 48 h after infestation with *Plutella xylostella* caterpillars (Px); *Brevicoryne brassicae* aphids (B); both *P. xylostella* and *B. brassica* simultaneously (Px+B), or without any herbivory (C)(for treatment coding also see Fig 1). Gene expression levels are shown as fold changes in mean relative expression compared to these in control plants (C). Bars present means ± SE (n=4).

**Table 2.** Regression analysis of the main effects of wild cabbage (*B. oleracea*) plant population (KIM, WIN, OH), herbivore treatment (C, B, Px and Px+B; see Fig 1), time point (6, 24, 48 h) and their interaction terms on the expression level of the JA-responsive defence marker gene *LOX* and the SA-responsive defence marker gene *PR-1* in Experiment 1

Experiment 1

| Tested gene | Factor | *N.d.f.* | *D.d.f* | *F* statistics | *P* value |
|---|---|---|---|---|---|
| *LOX* | Plant population (1) | 2 | 107 | 12.57 | **< 0.001** |
| | Treatment (2) | 3 | 107 | 15.16 | **< 0.001** |
| | Time point (3) | 2 | 107 | 36.08 | **< 0.001** |
| | Interaction 1*2 | 6 | 107 | 1.99 | 0.074 |
| | Interaction 1*3 | 4 | 107 | 6.78 | **< 0.001** |
| | Interaction 2*3 | 6 | 107 | 8.41 | **< 0.001** |
| | Interaction 1*2*3 | 12 | 107 | 1.10 | 0.368 |
| *PR-1* | Plant population (1) | 2 | 105 | 7.67 | **< 0.001** |
| | Treatment (2) | 3 | 105 | 7.07 | **< 0.001** |
| | Time point (3) | 2 | 105 | 3.07 | 0.051 |
| | Interaction 1*2 | 6 | 105 | 1.40 | 0.223 |
| | Interaction 1*3 | 4 | 105 | 4.99 | **0.001** |
| | Interaction 2*3 | 6 | 105 | 1.40 | 0.222 |
| | Interaction 1*2*3 | 12 | 105 | 1.15 | 0.331 |

## Experiment 2: effect of the order of arrival of *B. brassicae* aphids and *P. xylostella* caterpillars

In a first analysis including data of control plants (C) and plants infested with aphids (CB and B) alone (for treatment coding see Fig 1B), we confirmed the results of experiment 1. The expression levels of both *LOX* and *PR-1* were similar in plants infested by *B. brassicae* alone for a short period, i.e. 1 or 2 days (CB), or an extended period, i.e. 6 or 7 days (B) compared to control plants (C), irrespective of the plant population (Fig 3; Table 3a). In a second analysis, we investigated the effect of the temporal infestation order of *P. xylostella* caterpillars and *B. brassicae* aphids on gene expression levels.

### *LOX expression*

Herbivore treatment and plant population had a significant effect on the expression of *LOX* (Table 3b, Fig 3A-C). Overall, the presence of *B. brassicae* had relatively little effect on the expression of *LOX*, regardless of the order of arrival (CPx vs. BPx, $P$=0.72, Px vs. PxB, $P$=0.99, Fig 3A-C). *LOX* transcription differed only between plants that were infested with caterpillars first and aphids second and plants that were infested with caterpillars late, irrespective of whether there were also aphids on the plant (PxB vs. CPx, $P$=0.02; PxB vs. BPx, $P$=0.01). However, there was also a significant interaction between the time point of sampling and treatment. At both time points, *LOX* expression levels were equally high in plants that were infested with caterpillars late, irrespective of aphid presence (CPx-24h vs. CPx-48h and BPx-24h vs. BPx-48h, $P$>0.95). In plants infested with caterpillars early

and no aphids, *LOX* transcripts were significantly lower at 48 than at 24h (Px-24h vs. Px-48h, *P*=0.006), whereas in plants infested with caterpillars early and aphids late (PxB), transcription levels were equally low at both time points (*P*>0.05). The late infestation of *B. brassicae* and the extended period of caterpillar feeding tended to suppress the expression level of *LOX* (PxB-48h vs. Px-48h). *LOX* transcripts were higher in WIN than in KIM plants,

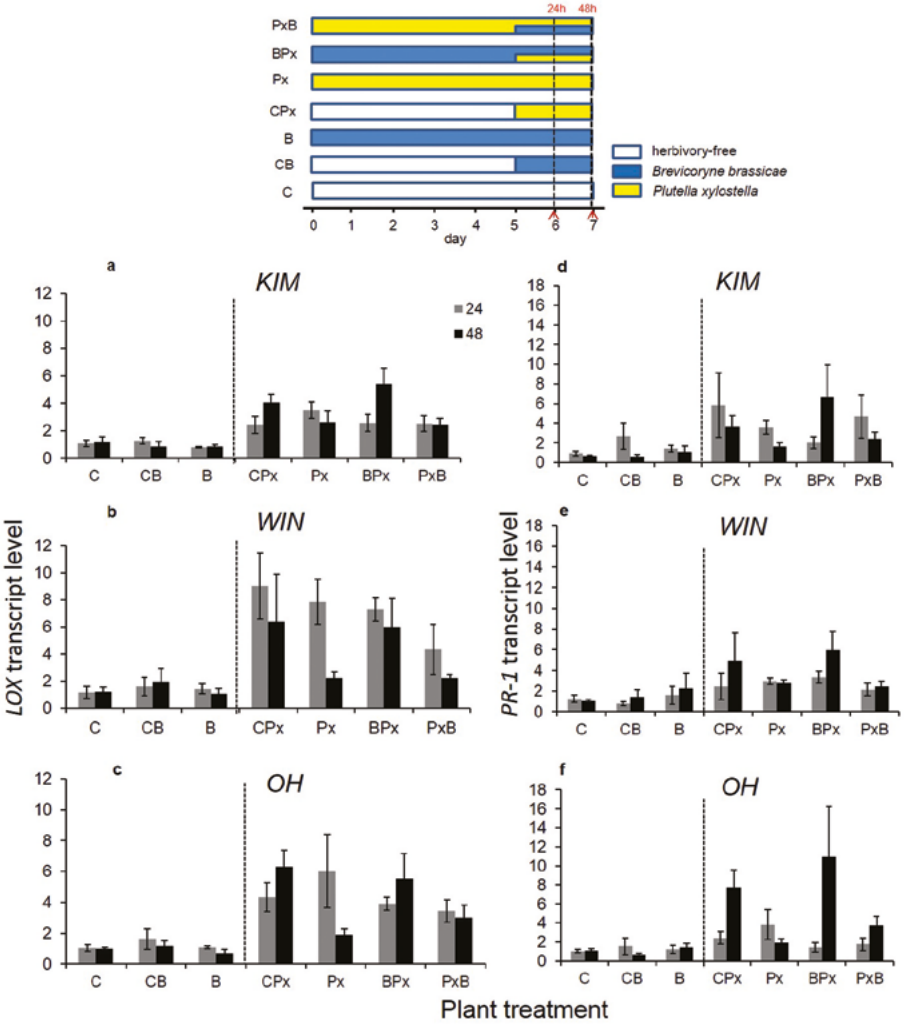


**Figure 3.** qRT-PCR analysis of transcript levels of the JA-responsive defence marker gene *LOX* (panels a-c) and a SA-responsive defence marker gene *PR-1* (panels D-F) in leaves of plants from three wild *Brassica oleracea* populations (KIM [A;D]; WIN [B;E]; OH [C;F]). Plants were infested with *Plutella xylostella* or *Brevicoryne brassicae* either at day 0 (Px and B) or day 5 (CPx and CB), or they were dually infested with *P. xylostella* at day 0 and with *B. brassicae* at day 5 (PxB), or with *B. brassicae* at day 0 and with *P. xylostella* at day 5 (BPx). Gene expression was measured 24 and 48h following treatment with the second herbivore (see also Fig 1). Gene expression levels are shown as fold changes in mean relative expression compared to these in herbivore free control plants (C). Bars present means ± SE (n=4).

whereas levels of this gene in OH plants did not differ from those in plants from the other two populations (Fig 2A-C, WIN vs. KIM, *P*=0.01, OH vs. KIM, *P*=0.17, and OH vs WIN, *P*=0.53). The effect of time point on *LOX* expression differed among plant populations. The up-regulation of *LOX* was fastest in WIN (levels were higher at 24 h than at 48 h, *P*=0.006) whereas for the other two populations there was no difference between transcript levels at 24 and 48h (KIM-24 vs. KIM-48, *P*=0.84, OH-24 vs. OH-48, *P*=0.99).

### *PR-1 expression*

The effect of herbivore treatment on *PR-1* transcription depended on the time of sampling (Table 3b, Fig 3D-F). *PR-1* transcript levels did not differ among the plant populations (Table 3b). In the treatments where caterpillars were introduced late, i.e. CPx and BPx, *PR-1* transcript levels were higher at 48 than at 24h, but this was only significant in the treatment where aphids were introduced first and caterpillars second (BPx-24 vs. BPx-48, *P*=0.05).

**Table 3.** Regression analysis of the main effects of plant population (KIM, WIN, OH), herbivore treatments (a [C, CB, B;]; b [CPx, BPx, Px, PxB; see Fig 1]), time point (24, 48 h) and their interaction terms on the expression level of the JA-responsive defence marker gene *LOX* and SA-responsive defence marker gene *PR-1* in Experiment 2

| Experiment 2(a) | Treatment of C, CB, B | | | | |
|---|---|---|---|---|---|
| Tested gene | Factor | *N.d.f.* | *D.d.f* | *F* statistics | *P* value |
| *LOX-2* | Plant population (1) | 2 | 53 | 0.3 | 0.739 |
| | Treatment (2) | 2 | 53 | 0.56 | 0.572 |
| | Time point (3) | 1 | 53 | 1.92 | 0.171 |
| | Interaction 1*2 | 4 | 53 | 0.14 | 0.968 |
| | Interaction 1*3 | 2 | 53 | 0.04 | 0.956 |
| | Interaction 2*3 | 2 | 53 | 0.53 | 0.593 |
| | Interaction 1*2*3 | 4 | 53 | 0.29 | 0.883 |
| *PR-1* | Factor | *N.d.f.* | | *F* statistics | *P* value |
| | Plant population (1) | 2 | 53 | 0.09 | 0.918 |
| | Treatment (2) | 2 | 53 | 0.75 | 0.479 |
| | Time point (3) | 1 | 53 | 0.71 | 0.403 |
| | Interaction 1*2 | 4 | 53 | 0.04 | 0.996 |
| | Interaction 1*3 | 2 | 53 | 1.54 | 0.224 |
| | Interaction 2*3 | 2 | 53 | 0.76 | 0.473 |
| | Interaction 1*2*3 | 4 | 53 | 0.6 | 0.664 |

| Experiment 2(b) | Treatment of CPx, Px, BPx,PxB | | | | |
|---|---|---|---|---|---|
| Tested gene | Factor | *N.d.f.* | *D.d.f* | *F* statistics | *P* value |
| *LOX* | Plant population (1) | 2 | 72 | 4.33 | **0.017** |
| | Treatment (2) | 3 | 72 | 4.57 | **0.005** |
| | Time point (3) | 1 | 72 | 3.31 | 0.073 |
| | Interaction 1*2 | 6 | 72 | 0.45 | 0.846 |
| | Interaction 1*3 | 2 | 72 | 6.02 | **0.004** |
| | Interaction 2*3 | 3 | 72 | 4.35 | **0.007** |
| | Interaction 1*2*3 | 6 | 72 | 0.45 | 0.845 |

| *PR-1* | Factor | *N.d.f.* | *D.d.f* | *F* statistics | *P* value |
|---|---|---|---|---|---|
| | Plant population (1) | 2 | 78 | 0.07 | 0.929 |
| | Treatment (2) | 3 | 78 | 0.74 | 0.532 |
| | Time point (3) | 1 | 78 | 5.17 | **0.026** |
| | Interaction 1*2 | 6 | 78 | 0.57 | 0.751 |
| | Interaction 1*3 | 2 | 78 | 1.71 | 0.187 |
| | Interaction 2*3 | 3 | 78 | 3.99 | **0.011** |
| | Interaction 1*2*3 | 6 | 78 | 0.52 | 0.793 |

### Experiment 3: effect of dual infestation with *B. brassicae* aphids and caterpillars of different herbivore species

#### *LOX expression*

The extent to which *LOX* was upregulated was affected by caterpillar species, plant population and the time of sampling, while it was not affected by the presence or absence of aphid feeding (Table 4; Fig 4A-C). *LOX* transcript levels were highest in plants infested with *M. brassicae*, intermediate in plants infested with *P. xylostella* and lowest in plants infested with *P. brassicae* (Fig 4A-C; Mb vs. Pb, $P<0.001$, Mb vs. Px, $P=0.03$, Px vs. Pb, $P<0.001$). Overall, transcript levels of *LOX* were higher in KIM and OH plants than in WIN plants (KIM vs. OH, $P=0.18$, WIN vs. KIM and WIN vs. OH, $P<0.001$) and they were higher at 48 h than at 24 h after initiation of caterpillar feeding. However, the extent to which transcripts increased depended on the population; whereas transcript levels were similar at 24 h following the introduction of the herbivores (all comparisons $P>0.05$), transcript levels at 48h were highest in KIM, intermediate in OH, and lowest in WIN (KIM vs. OH, $P=0.005$, KIM vs. WIN and OH vs. WIN, $P<0.001$).

#### *PR-1 expression*

The results for expression levels of *PR-1* in response to feeding by different caterpillar species in the presence or absence of aphids were more idiosyncratic; two of the four three-way interactions were significant (Table 4, Fig 4D-F). Overall, *PR-1* transcripts increased more in response to *M. brassicae* than to *P. xylostella* feeding ($P=0.002$), whereas transcription of this gene was similar in response to *P. brassicae* feeding and feeding by the other two caterpillar species (Pb vs. Px and Pb vs. Mb, $P>0.05$). Early aphid infestation did not affect transcript levels of *PR-1* in response to *P. xylostella* and *M. brassicae* feeding whereas it has a tendency to decrease *PR-1* transcript levels in plants infested with *P. brassicae* larvae for 48 h; however, this was not statistically significant. Overall expression levels of *PR-1* in OH plants were higher than in the other two populations (OH vs. KIM, $P<0.001$, OH vs. WIN, $P=0.04$, KIM vs. WIN, $P=0.46$). Expression levels of *PR-1* were higher at 48 than at 24h following the introduction of the caterpillars, but the extent of this increase was plant-population dependent.

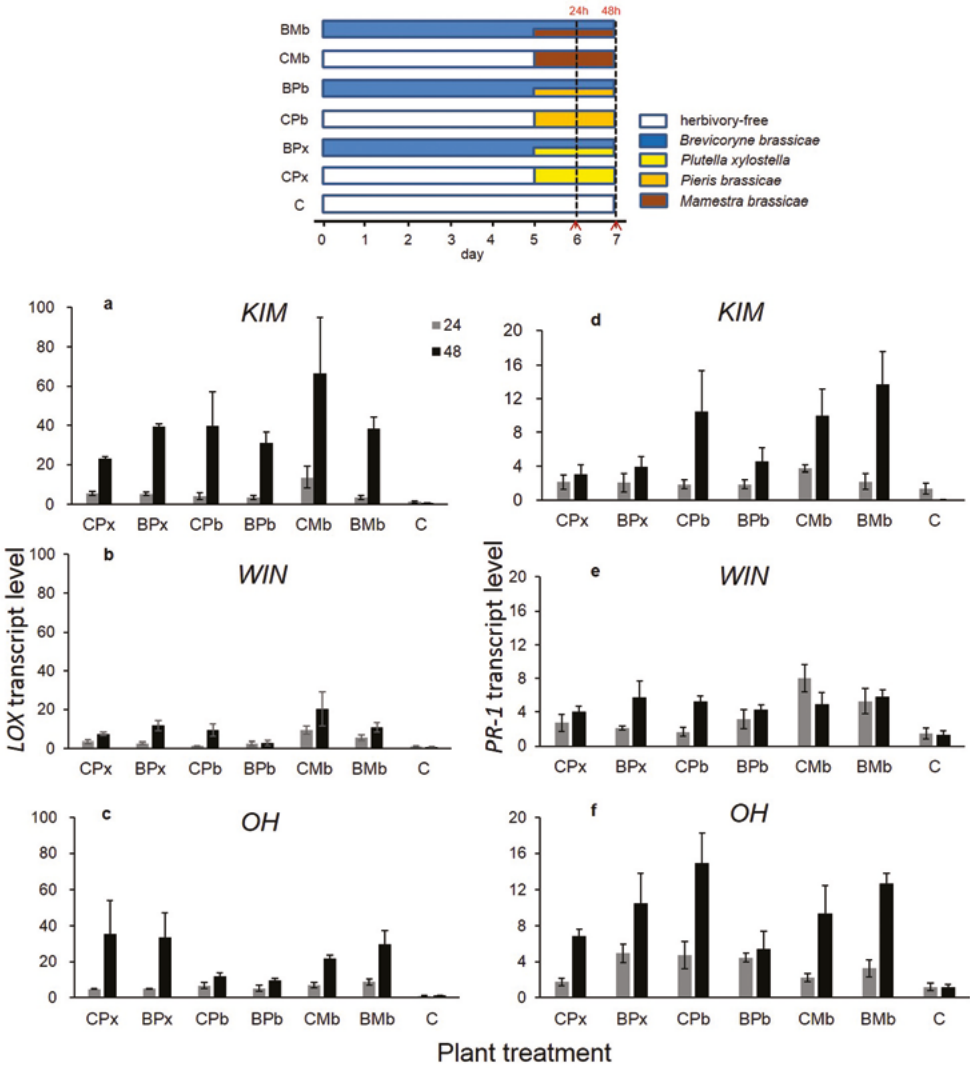


**Figure 4.** qRT-PCR analysis of transcript levels of the JA-responsive defence marker gene *LOX* (panels A-C) and a SA-responsive defence marker gene *PR-1* (panels D-F) in leaves of wild *Brassica oleracea* populations (KIM [A;D]; WIN [B;E]; OH [C;F]). Plants were infested by caterpillars of one of three lepidopteran species *Plutella xylostella* (CPx), *Pieris brassicae* (CPb) or *Mamestra brassicae* (CMb) on day 5, or they were dually infested with *Brevicoryne brassicae* at day 0 and caterpillars on day 5 (BPx, BPb and BMb, respectively). Gene expression was measured 24 and 48h following treatment with the second herbivore (see also Fig 1). Gene expression levels are shown as fold changes in mean relative expression compared to these in herbivore free control plants (C). Bars present means ± SE (n=4).

**Table 4.** Regression analysis of the main effects of plant population (KIM, WIN, OH), herbivore treatment (C, CPx, BPx, CPb, BPb, CMb, BMb; see Fig 1), time point (24, 48 h) and their interaction terms on the expression level of JA-responsive defence marker gene *LOX* and SA- responsive defence marker gene *PR-1* in Experiment 3

Experiment 3

| Tested gene | Factor | *N.d.f.* | *D.d.f* | *F* statistics | *P* value |
|---|---|---|---|---|---|
| *LOX* | Plant population (1) | 2 | 108 | 37.69 | **< 0.001** |
| | caterpillar infestation (2) | 2 | 108 | 20.98 | **< 0.001** |
| | *B. brassicae* infestation (3) | 1 | 108 | 2.82 | 0.096 |
| | Time point (4) | 1 | 108 | 190.11 | **< 0.001** |
| | Interaction 1*2 | 4 | 108 | 2.17 | 0.077 |
| | Interaction 1*3 | 2 | 108 | 0.90 | 0.41 |
| | Interaction 2*3 | 2 | 108 | 2.50 | 0.087 |
| | Interaction 1*4 | 2 | 108 | 12.39 | **< 0.001** |
| | Interaction 2*4 | 2 | 108 | 0.81 | 0.45 |
| | Interaction 3*4 | 1 | 108 | 0.32 | 0.57 |
| | Interaction 1*2*3 | 4 | 108 | 1.66 | 0.16 |
| | Interaction 1*2*4 | 4 | 108 | 2.00 | 0.10 |
| | Interaction 1*3*4 | 2 | 108 | 1.47 | 0.23 |
| | Interaction 2*3*4 | 2 | 108 | 2.65 | 0.075 |
| | Interaction 1*2*3*4 | 4 | 108 | 1.25 | 0.30 |
| *PR-1* | Factor | *N.d.f.* | *D.d.f* | *F* statistics | *P* value |
| | Plant population (1) | 2 | 108 | 6.83 | **0.002** |
| | caterpillar infestation (2) | 2 | 108 | 6.30 | **0.003** |
| | *B. brassicae* infestation (3) | 1 | 108 | 0.07 | 0.799 |
| | Time point (4) | 1 | 108 | 59.87 | **< 0.001** |
| | Interaction 1*2 | 4 | 108 | 2.43 | 0.052 |
| | Interaction 1*3 | 2 | 108 | 1.35 | 0.263 |
| | Interaction 2*3 | 2 | 108 | 1.78 | 0.174 |
| | Interaction 1*4 | 2 | 108 | 3.42 | **0.036** |
| | Interaction 2*4 | 2 | 108 | 0.05 | 0.948 |
| | Interaction 3*4 | 1 | 108 | 0.11 | 0.742 |
| | Interaction 1*2*3 | 4 | 108 | 1.40 | 0.238 |
| | Interaction 1*2*4 | 4 | 108 | 2.66 | **0.036** |
| | Interaction 1*3*4 | 2 | 108 | 1.14 | 0.324 |
| | Interaction 2*3*4 | 2 | 108 | 5.01 | **0.008** |
| | Interaction 1*2*3*4 | 4 | 108 | 0.10 | 0.983 |



## Discussion

Both marker genes, *LOX* and *PR-1* were up-regulated in response to single *P. xylostella* infestation in all three cabbage populations, whereas single infestation by *B. brassicae* aphids did not affect transcription levels of either of these two genes. In addition, dual infestation with aphids and *P. xylostella* caterpillars, simultaneously or separated in time (regardless of the order of infestation) had little or no effect on transcription levels of *LOX* and *PR-1*. Caterpillar species differentially affected upregulation of the two marker genes. As was found for *P. xylostella*, aphid presence did not interfere with transcription

of *LOX* and *PR-1* in response to feeding by *P. brassicae* or *M. brassicae* caterpillars. The main effects were consistent across the three cabbage populations, though there were population-related differences in the temporal dynamics of *LOX* and *PR-1* transcription, in the response to the three caterpillar species and also in the extent to which plants of the three populations upregulated gene expression in response to the various herbivore treatments.

Based on negative SA-JA crosstalk, we hypothesized that aphid infestation would lead to higher expression levels of SA-responsive genes and would suppress the transcription of JA-responsive genes in response to caterpillar attack. The temporal order of herbivore attack can further influence the timing and intensity of plant defence responses to aphid and caterpillar feeding and their interaction (Erb et al. 2011; Stam et al. 2014). In contrast to our hypothesis, the results of the present study show no effects of aphid infestation on the transcript levels of a JA- and SA-responsive gene when plants were challenged by both *B. brassicae* and different caterpillar species, irrespective of the temporal sequence of aphid and caterpillar attack. The lack of interference with transcription of JA- or SA-responsive genes by aphid infestation on the *B. oleracea* plants may be attributed to: 1) a lack of effects of aphid infestation on SA production, as was also reported by Ali and Agrawal (2014) for *Asclepias tuberosa*. Low transcript levels of *PR-1* in the present study may imply overall low activation of SA signalling in *B. oleracea* plants in response to aphid infestation. 2) Absence of negative crosstalk between SA and JA in *B. oleracea*. A review by Thaler *et al.* (2012) reported the absence of SA-JA antagonism in several plant species, e.g. *Zea mays* (Poaceae), *Asclepias exaltata* (Apocynaceae) and *Picea abies* (Pinaceae), suggesting that this phenomenon is not ubiquitous across taxa even when they are in the same family like *A. thaliana* and *B. oleracea*. 3) The temporal kinetics of JA- and SA-mediated defence induction and concomitant gene expression in wild cabbage may differ from those reported for the model plant *Arabidopsis thaliana*. 4) In wild *B. oleracea*, other genes than *LOX* and *PR-1* are involved in JA-SA antagonism.

Aphid (*B. brassicae*) feeding alone had no effect on the expression levels of both *PR-1*, a gene supposedly responsive to aphid feeding (De Vos et al. 2005) and *LOX*. This is consistent with data by Moran and Thompson (2001) who reported that *B. brassicae* did not induce responses associated with JA- or SA-related metabolic processes in *A. thaliana* plants. Due to the stealthy feeding style of aphids and the minimal wounding they cause to plants, induction of SA in response to aphid herbivory occurs very locally, i.e. only at the site of aphid feeding, and, therefore, transcript levels of *PR-1* may be low and difficult to detect (De Vos et al. 2005). Previous studies have reported that the saliva of aphids is rich in elicitors (Hogenhout and Bos 2011; Walling 2000). Both SA- and JA-responsive genes reacted to feeding by *B. brassicae* and *M. persicae* on Arabidopsis (Kusnierczyk et al. 2011; Kusnierczyk et al. 2008; Moran and Thompson 2001). However, the aphid-induced responses of plants can also be aphid species-specific. For instance, *M. persicae* feeding

induced SA-responsive genes in *A. thaliana*, whereas *B. brassicae* did not (Appel et al. 2014). *Brevicoryne brassicae* may have evolved to avoid inducing defensive responses in wild cabbage plants that is often attacked by this aphid in its natural habitat (Newton et al. 2009a) or it may manipulate the plant defence for its own benefit (De Vos et al. 2007; Walling 2008). This has also been reported for a population of the herbivorous spider mite *Tetranychus urticae* that does not induce JA-regulated defences in tomato that negatively affect spider mite performance (Kant et al. 2008).

Feeding by *P. xylostella* caterpillars significantly up-regulated gene expression not only of *LOX*, but also of *PR-1*, irrespective of whether the caterpillars were feeding alone or together with aphids. Studies by Kroes *et al.* (2015) and Ehlting *et al.* (2008) showed similar results, confirming that *P. xylostella* induces the expression of both SA- and JA-responsive genes in Arabidopsis. Glucose oxidase, present in the saliva of *Helicoverpa zea* caterpillars, induces SA signalling which leads to inhibition of JA signalling and eventually prevents the induction of nicotine in *Nicotiana tabacum* plants (Musser et al. 2005; Musser et al. 2002). In our study, not only infestation by *P. xylostella*, but also by two other lepidopteran species, *P. brassicae* and *M. brassicae,* consistently up-regulated the expression of both *LOX* and *PR-1* genes on all three cabbage populations, regardless of the differences in dietary specialization and salivary elicitors (Felton 2008). In previous studies, it was shown that both *P. xylostella* and *P. brassicae*, but not *M. brassicae* gained fitness benefits by feeding on wild and cultivated cabbage plants co-infested with *B. brassicae* aphids (Li et al. 2014; Soler et al. 2012). However, as we did not record an effect of aphid infestation on the transcription of the JA-responsive gene *LOX*, it remains to be investigated whether the enhanced performance of *P. xylostella* and *P. brassicae* (Li et al. 2014; Soler et al. 2012) result from attenuation of JA-mediated defences. Alternatively, negative interference between JA and SA may affect other genes than *LOX* and *PR-1* in wild *B. oleracea*.

The extent to which *LOX* and *PR-1* were upregulated differed among plants infested by different species of caterpillars: infestation with *M. brassicae* up-regulated both genes more compared to infestation by *P. brassicae* or *P. xylostella*. Herbivores with a similar feeding mode tend to induce more similar transcriptome responses in *A. thaliana* plants than herbivores with a different feeding mode (Appel et al. 2014; Bidart-Bouzat and Kliebenstein 2011; Ehlting et al. 2008). However, for transcriptional responses induced by the chewing herbivores *P. xylostella* or *P. rapae*, only 32% to 40% of the genes were elicited commonly (Ehlting et al. 2008). Thus, induction of signal transduction components in plants may differ among herbivore species, even when the attacking herbivore species are from the same feeding guild (Bidart-Bouzat and Kliebenstein 2011; Diezel et al. 2009; Mewis et al. 2006). Although the chewing herbivores in this study are all members of the Lepidoptera, they differ in their feeding behaviour. First and second instar *P. xylostella* larvae usually mine the leaf spongy mesophyll tissues, while later instar larvae feed on the abaxial surface of leaves often leaving the upper epidermis intact (Sarfraz et al. 2005). *Pieris brassicae* larvae chew

the leaf tissues gregariously, and initially cause a single damage site on the leaf. *Mamestra brassicae* larvae disperse immediately after egg hatching and then feed solitarily causing scattered sites of feeding damage on the leaves. It remains unknown to what extent the different feeding patterns of these caterpillars contribute to the differences in induced plant transcriptional responses.

At the plant population level, we found differences in the overall transcriptional responses of plants to the various treatments and also in the temporal dynamics of these responses. These population-related differences were not consistent among the various experiments. This suggests that variation in conditions that could not be controlled, either related to the greenhouse environment or the plants themselves, resulted in population-specific variation in the response to the various treatments. These results reveal that the expression of genes involved in JA and SA defence signalling can be quite subtle and linking gene expression to responses occurring at a higher level of biological organization should be done cautiously. Nevertheless, also at the population level, there is no evidence to support JA-SA antagonism based on expression levels of the two marker genes.

The non-interactive effects of aphid and caterpillar infestation on the transcription levels of JA- and SA- responsive marker genes in the wild cabbage populations, regardless of the temporal sequence of both types of herbivory, implies that JA-SA antagonism may not ubiquitously occur in all plant taxa. The interaction between the JA and SA signalling pathways is likely to be far more complex involving various genes of which some interact antagonistically and others don't.

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## References

Agrawal AA (1999) Induced plant defense: evolution of induction and adaptive phenotypic plasticity. In Agrawal AA, Tuzun S, Bent E (Eds.) *Induced Plant Defenses Against Pathogens and Herbivores. Biochemstry, Ecology and Agriculture* (pp. 251-268). St. Paul, Minosota: APS Press.

Appel HM et al. (2014) Transcriptional responses of *Arabidopsis thaliana* to chewing and sucking insect herbivores. Frontiers in Plant Science 5: 1-20.

Bell E, Creelman RA, Mullet JE (1995) A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in Arabidopsis. Proceedings of the National Academy of Sciences USA 92: 8675-8679.

Bidart-Bouzat MG, Kliebenstein D (2011) An ecological genomic approach challenging the paradigm of differential plant responses to specialist versus generalist insect herbivores. Oecologia 167: 677-689.

De Vos M, Kim JH, Jander G (2007) Biochemistry and molecular biology of Arabidopsis-aphid interactions. Bioessays 29: 871-883.

De Vos M et al. (2005) Signal signature and transcriptome changes of Arabidopsis during pathogen and insect attack. Molecular Plant-Microbe Interactions 18: 923-937.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends in Plant Science 15: 167-175.

Diezel C, von Dahl CC, Gaquerel E, Baldwin IT (2009) Different lepidopteran elicitors account for cross-talk in herbivory-induced phytohormone signaling. Plant Physiology 150: 1576-1586.

Ehlting J, Chowrira SG, Mattheus N, Aeschliman DS, Arimura G, Bohlmann J (2008) Comparative transcriptome analysis of *Arabidopsis thaliana* infested by diamond back moth (*Plutella xylostella*) larvae reveals signatures of stress response, secondary metabolism, and signalling. BMC Genomics 9: 20.

Erb M, Meldau S, Howe GA (2012) Role of phytohormones in insect-specific plant reactions. Trends in Plant Science 17: 250-259.

Erb M, Robert CAM, Hibbard BE, Turlings TCJ (2011) Sequence of arrival determines plant-mediated interactions between herbivores. Journal of Ecology 99: 7-15.

Felton GW (2008) Caterpillar secretions and induced plant responses. Springer, Hohenheim, Germany.

Gols R, Wagenaar R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM, Harvey JA (2008) Genetic variation in defense chemistry in wild cabbages affects herbivores and their endoparasitoids. Ecology 89: 1616-1626.

Harvey JA, van Dam NM, Raaijmakers CE, Bullock JM, Gols R (2011) Tri-trophic effects of inter- and intra-population variation in defence chemistry of wild cabbage (Brassica oleracea). Oecologia 166: 421-431.

Harvey JA, Witjes LMA, Benkirane M, Duyts H, Wagenaar R (2007) Nutritional suitability and ecological relevance of *Arabidopsis thaliana* and *Brassica oleracea* as foodplants for the cabbage butterfly, Pieris rapae. Plant Ecology 189: 117-126.

Hogenhout SA, Bos JI (2011) Effector proteins that modulate plant-insect interactions. Current Opinion in Plant Biology 14: 422-428.

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annual Review of Plant Biology 59: 41-66.

Jirage D, Zhou N, Cooper B, Clarke JD, Dong XN, Glazebrook J (2001) Constitutive salicylic acid-dependent signaling in *cpr1* and *cpr6* mutants requires PAD4. The Plant Journal 26: 395-407.

Kant MR, Sabelis MW, Haring MA, Schuurink RC (2008) Intraspecific variation in a generalist herbivore accounts for differential induction and impact of host plant defences. Proceedings of the Royal Society of London B: Biological Sciences 275: 443-452.

Karban R, Baldwin I (1997) Induced responses to herbivory University of Chicago Press. Chicago, Illinois, USA.

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annual Review of Plant Biology 53: 299-328.

Kliebenstein DJ, Kroymann J, Brown P, Figuth A, Pedersen D, Gershenzon J, Mitchell-Olds T (2001) Genetic control of natural variation in Arabidopsis glucosinolate accumulation. Plant Physiology 126: 811-825.

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiology 146: 839-844.

Kroes A, van Loon JJA, Dicke M (2015) Density-dependent interference of aphids with caterpillar-induced defenses in Arabidopsis: involvement of phytohormones and transcription factors. Plant and Cell Physiology 56: 98-106.

Kusnierczyk A, Tran DHT, Winge P, Jorstad TS, Reese JC, Troczynska J, Bones AM (2011) Testing the importance of jasmonate signalling in induction of plant defences upon cabbage aphid (*Brevicoryne brassicae*) attack. BMC Genomics 12: 16.

Kusnierczyk A, Winge P, Jorstad TS, Troczynska J, Rossiter JT, Bones AM (2008) Towards global understanding of plant defence against aphids--timing and dynamics of early Arabidopsis defence responses to cabbage aphid (*Brevicoryne brassicae*) attack. Plant, Cell and Environment 31: 1097-1115.

Li Y, Dicke M, Harvey J, Gols R (2014) Intra-specific variation in wild *Brassica oleracea* for aphid-induced plant responses and consequences for caterpillar-parasitoid interactions. Oecologia 174: 853-862.

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2−ΔΔCT method. Methods 25: 402-408.

Mewis I, Appel HM, Hom A, Raina R, Schultz JC (2005) Major signaling pathways modulate Arabidopsis glucosinolate accumulation and response to both phloem-feeding and chewing insects. Plant Physiology 138: 1149-1162.

Mewis I, Tokuhisa JG, Schultz JC, Appel HM, Ulrichs C, Gershenzon J (2006) Gene expression and glucosinolate accumulation in *Arabidopsis thaliana* in response to generalist and specialist herbivores of different feeding guilds and the role of defense signaling pathways. Phytochemistry 67: 2450-2462.

Moran PJ, Thompson GA (2001) Molecular responses to aphid feeding in Arabidopsis in relation to plant defense pathways. Plant Physiology 125: 1074-1085.

Mouttet R, Kaplan I, Bearez P, Amiens-Desneux E, Desneux N (2013) Spatiotemporal patterns of induced resistance and susceptibility linking diverse plant parasites. Oecologia 173: 1379-1386.

Musser RO, Cipollini DF, Hum-Musser SM, Williams SA, Brown JK, Felton GW (2005) Evidence that the caterpillar salivary enzyme glucose oxidase provides herbivore offense in Solanaceous plants. Archives of Insect Biochemistry and Physiology 58: 128-137.

Musser RO, Hum-Musser SM, Eichenseer H, Peiffer M, Ervin G, Murphy JB, Felton GW (2002) Herbivory: caterpillar saliva beats plant defences. Nature 416: 599-600.

Newton EL, Bullock JM, Hodgson D (2009a) Bottom-up effects of glucosinolate variation on aphid colony dynamics in wild cabbage populations. Ecological Entomology 34: 614-623.

Newton EL, Bullock JM, Hodgson DJ (2009b) Glucosinolate polymorphism in wild cabbage (*Brassica oleracea*) influences the structure of herbivore communities. Oecologia 160: 63-76.

Pieterse CM, Leon-Reyes A, Van der Ent S, Van Wees SC (2009) Networking by small-molecule hormones in plant immunity. Nature Chemical Biology 5: 308-316.

Pieterse CMJ, Dicke M (2007) Plant interactions with microbes and insects: from molecular mechanisms to ecology. Trends in Plant Science 12: 564-569.

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal modulation of plant immunity. Annual Review of Cell and Developmental Biology 28: 28.21-28.33.

Poelman EH, Zheng SJ, Zhang Z, Heemskerk NM, Cortesero AM, Dicke M (2011) Parasitoid-specific induction of plant responses to parasitized herbivores affects colonization by subsequent herbivores. Proceedings of the National Academy of Sciences USA 108: 19647-19652.

Rodriguez-Saona CR, Musser RO, Vogel H, Hum-Musser SM, Thaler JS (2010) Molecular, biochemical, and organismal analyses of tomato plants simultaneously attacked by herbivores from two feeding guilds. Journal of Chemical Ecology 36: 1043-1057.

Sarfraz M, Keddie AB, Dosdall LM (2005) Biological control of the diamondback moth, *Plutella xylostella*: a review. Biocontrol Science and Technology 15: 763-789.

Schmidt DD, Voelckel C, Hartl M, Schmidt S, Baldwin IT (2005) Specificity in ecological interactions. Attack from the same lepidopteran herbivore results in species-specific transcriptional responses in two solanaceous host plants. Plant Physiology 138: 1763-1773.

Schoonhoven LM, van Loon JJA, Dicke M (2005) Insect-Plant Biology. 2nd edn. Oxford University Press, Oxford.

Soler R, Badenes-Perez FR, Broekgaarden C, Zheng SJ, David A, Boland W, Dicke M (2012) Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. Functional Ecology 26: 156-166.

Stam JM, Kroes A, Li Y, Gols R, van Loon JJA, Poelman EH, Dicke M (2014) Plant interactions with multiple insect herbivores: from community to genes. Annual Review of Plant Biology 65: 689-713.

Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science 17: 260-270.

Traw M, Kim J, Enright S, Cipollini D, Bergelson J (2003) Negative cross-talk between salicylate- and jasmonate-mediated pathways in the Wassilewskija ecotype of *Arabidopsis thaliana*. Molecular Ecology 12: 1125-1135.

Voelckel C, Baldwin IT (2004) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. The Plant Journal 38: 650-663.

Walling LL (2000) The myriad plant responses to herbivores. Journal of Plant Growth Regulation 19: 195-216.

Walling LL (2008) Avoiding effective defenses: Strategies employed by phloem-feeding insects. Plant Physiology 146: 859-866.

Wu J, Baldwin IT (2009) Herbivory-induced signalling in plants: perception and action. Plant, Cell and Environment 32: 1161-1174.

Wu J, Baldwin IT (2010) New insights into plant responses to the attack from insect herbivores. Annual Review of Genetics 44: 1-24.

Wu J, Hettenhausen C, Schuman MC, Baldwin IT (2008) A comparison of two *Nicotiana attenuata* accessions reveals large differences in signaling induced by oral secretions of the specialist herbivore *Manduca sexta*. Plant Physiology 146: 927-939.

Zarate SI, Kempema LA, Walling LL (2007) Silverleaf whitefly induces salicylic acid defenses and suppresses effectual jasmonic acid defenses. Plant Physiology 143: 866-875.

Zhang PJ, Zheng SJ, van Loon JJA, Boland W, David A, Mumm R, Dicke M (2009) Whiteflies interfere with indirect plant defense against spider mites in Lima bean. Proceedings of the National Academy of Sciences USA 106: 21202-21207.

Zhu F, Broekgaarden C, Weldegergis BT, Harvey JA, Vosman B, Dicke M, Poelman EH (2015) Parasitism overrides herbivore identity allowing hyperparasitoids to locate their parasitoid host using herbivore-induced plant volatiles. Molecular Ecology 24: 2886-2899.

# General discussion

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Chapter 7

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## Introduction

Plants and insects are the most abundant and diverse groups of organisms on Earth and they share a long co-evolutionary history (Schoonhoven et al., 2005). The study of interactions between plants and insects at different levels of biological organization, from molecular mechanisms to processes structuring ecological communities, has provided opportunities for an integrated research approach (Kessler and Baldwin, 2002; Schoonhoven et al., 2005; Zheng and Dicke, 2008). Plants respond to herbivore attack with changes in chemical, physiological and/or morphological traits, that prevent or reduce the growth and/or survival of the herbivores (Karban and Baldwin, 1997). Over the last decades, research has made progress in revealing mechanisms that underlie plant defences against herbivores by studying many different plant-insect systems within a tritrophic context.

During their life time, plants may be under attack by tens to hundreds of herbivore species (Agrawal and Karban, 1999; Zheng and Dicke, 2008). To cope with the diversity of attackers, plants have evolved a variety of defences, including constitutive and induced defences (Walling, 2000; Kessler and Baldwin, 2002; Schoonhoven et al., 2005; Stam et al., 2014). Constitutive defences are present independent of attack. In addition, inducible defences allow plants to respond adequately and efficiently to the actual presence of attackers, and these are often specific for the attacking species (Agrawal and Karban, 1999; Howe and Jander, 2008). Chemical and morphological traits that negatively affect the performance / survival of herbivores are referred to as plant direct defences (Schoonhoven et al., 2005; Kessler and Halitschke, 2007). Plants also emit volatiles when challenged by insect herbivores, often referred to as herbivore-induced plant volatiles (HIPV), that attract the natural enemies of the herbivores(Turlings et al., 1990; Vet and Dicke, 1992). The enhancement of the activity of natural enemies to control insect herbivores functions as an indirect defence.

Specificity of inducible plant defences is mediated by chemical elicitors present in insect oral secretions and mechanical wounding caused by the herbivore. Moreover, the feeding guild of herbivores, i.e. leaf chewing or phloem sucking, as well as their dietary specialization, i.e. being a generalist or a specialist, are important factors that can affect the nature of induced plant defences (Voelckel and Baldwin, 2004; Bidart-Bouzat and Kliebenstein, 2011; Ali and Agrawal, 2012). Altered plant traits in response to herbivory may subsequently affect the behaviour and performance of any insect that interacts with these plants (Ohgushi, 2005) and as a result affect the composition and structure of the entire insect community (Poelman et al., 2008; Utsumi and Ohgushi, 2008; Dicke and Baldwin, 2010).

Within a species, plant populations often display variation in constitutive and induced defences to the same type of herbivory (Rapusas et al., 1996; Gols et al., 2008). Such intraspecific variation in plant defensive traits is hypothesized to result from selection pressure by the local biotic and abiotic environment (Briggs and Walters, 1997; Alonso-

Blanco et al., 2009). Insect herbivory may drive evolution of plant resistance and competitive ability in the field (Agrawal et al., 2012). For instance, the wild cabbage populations used in this thesis have been shown to differ in glucosinolate profiles, important secondary metabolites in brassicaceous plants, and to harbour different herbivore densities (Moyes et al., 2000; Moyes and Raybould, 2001; Newton et al., 2009). The investigation of natural variation in how plants interact with their insect community is important to understand the evolution of plant defences, *i.e.* how selection pressures drive adaptation of plants to environmental variation (Alonso-Blanco and Koornneef, 2000; Alonso-Blanco et al., 2009).

The phytohormones jasmonic acid (JA) and salicylic acid (SA) play major roles in regulating both plant direct and indirect induced defences against herbivorous insects (Howe and Jander, 2008; Pieterse et al., 2012). Plants have been shown to predominantly induce JA-dependent defences in response to attack by leaf-chewing herbivores and mainly induce SA-dependent responses in response to phloem-feeding herbivores (Erb et al., 2012; Pieterse et al., 2012). Both SA and JA are involved in the biosynthesis of HIPV (Ozawa et al., 2000; van Poecke and Dicke, 2002), but JA is the most important regulator of HIPV when plants are under attack by leaf-chewing herbivores (Heil, 2008; Dicke et al., 2009). Furthermore, the well-established antagonism between SA and JA defence signalling may hinder the plant's ability to optimally defend against both types of attackers when present on the plant simultaneously. For example, the plant's response may be prioritized as a result of the activation by a specific phytohormone. Moreover, the activation of defence pathways also depends on the temporal dynamics of the attack by different herbivore species and the kinetics of the plant's response to the challenge (Walling, 2000; Koornneef and Pieterse, 2008).

Although a number of studies have assessed the effects of direct and indirect plant defences on tri-trophic interactions involving a plant, one herbivore and one parasitoid, little is known about the effect of multiple herbivory on induced plant defence traits and how these affect tri-trophic interactions. Furthermore, it remains unknown how similar plant phenotypic changes are among plant populations when induced by the same types of herbivory. Studies on plant-trait mediated interactions with insects should be extended from systems with single species at each trophic level to systems where multiple herbivore species and their natural enemies interact. The main aim of this thesis project was to explore how plants respond to dual herbivory by phloem-sucking and leaf-chewing insects, and what the consequences are for the interactions between herbivores and their parasitoids, as well as for the entire associated insect community.

Here, I integrated plant responses to multiple herbivory by insects belonging to different feeding guilds at different levels of biological organisation: plant transcriptional responses at the molecular level (Chapter 6), plant volatile chemistry (=secondary metabolism) (Chapter 5), consequences of plant direct and indirect defences for insect individuals (Chapter 4

& 5) and the entire community (Chapter 3). Using three wild cabbage populations that differ in both constitutive and inducible chemical defences, I have investigated 1) how co-infestation of aphids (*Brevicoryne brassicae*) and caterpillars (*Plutella xylostella*, *Mamestra brassicae* and *Pieris brassicae*) may interfere with the activation of SA- and JA signalling pathways (Chapter 6); 2) how initial aphid infestation may alter volatile blends emitted by caterpillar-infested plants (Chapter 5); 3) how aphid infestation affects food plant quality for caterpillars and their parasitoids (Chapter 4), and the relative attraction of the two parasitoids to blends emitted by dually-infested plants and plants infested with hosts alone (Chapter 5) and 4) how early-season aphid infestation influences the composition of the associated insect community throughout the season (Chapter 3).

## How do aphid and caterpillar infestation affect plant phenotypic changes?

### Plant perspective

Our data on transcription levels of marker genes of the two major defence signal-transduction pathways did not provide support for SA-JA antagonism (Chapter 6). Compared to single infestation with each of the two herbivores, dual infestation with aphids and caterpillars had no interactive effect on transcription levels of the SA-responsive marker gene *PR-1* and the JA-responsive marker gene *LOX,* regardless of 1) the temporal sequence of aphid and caterpillar attack and 2) the identity of the attacking caterpillar species. Both *PR-1* and *LOX* were up-regulated by caterpillar feeding, but neither of them was induced in response to aphid infestation (Chapter 6). Aphid infestation in this study system may only induce limited SA production, which may explain the observed non-interactive effect of dual infestation on the activation of SA- and JA-signalling pathways in wild *B. oleracea* plants. We selected the JA- and SA-responsive marker genes based on previous studies using Arabidopsis. However, other genes than *LOX* and *PR-1* may be involved in the induction of JA and SA signalling pathways in wild *B. oleracea*. Therefore, investigating the transcription of multiple hormone signalling-related genes is required to investigate SA-JA antagonism in *B. oleracea*. Although several studies have provided experimental evidence for SA-JA antagonism in the model plant *Arabidopsis thaliana*, including those using microarray analysis of all genes (Beckers and Spoel, 2006; Koornneef and Pieterse, 2008; Zhang et al., 2013), less is known about *B. oleracea*.

I also assessed how co-infestation with aphids affects the volatile profiles of caterpillar-challenged plants. Using multivariate statistics, the volatile blends emitted by plants infested with hosts alone and by plants dually infested with hosts and aphids could not be separated (Chapter 5), suggesting that the effect of SA-JA antagonism plays a minor role

in the volatile emission of plants with dual infestation. However, the univariate statistics showed that aphid infestation reduced the emission rate of most volatile compounds induced by caterpillar infestation. Reduced volatile emission was more pronounced in plants infested with aphids for a longer time period (Chapter 5). This result suggests that aphid infestation negatively interfered with caterpillar-induced volatile emission, and shows that aphids do interfere with caterpillar-induced plant responses. Such results have been recorded for other study systems as well (Zhang et al., 2009; Zhang et al., 2013). What the mechanism of interference is in my study system remains to be investigated. Plant volatile biosynthesis in response to multiple herbivory may be regulated by the interaction of various plant signalling pathways in a much more complex fashion than SA-JA antagonism (Engelberth et al., 2001; Hare, 2011). However, given the complexity of plant transcriptional responses to herbivory, a variety of experimental factors could additionally influence the response of plants. Plant and insect developmental stage, herbivore density and treatment duration are among the factors that can affect transcriptional responses (Erb et al., 2012). To gain a better understanding of mechanisms underlying the effects of multiple herbivory on plant indirect responses, future studies should combine the analysis of volatile chemistry with activity of plant signalling pathways / transcription factors by using *e.g.* microarray analysis (Voelckel and Baldwin, 2004; Zhang et al., 2013).

**Insect perspective**

In Chapter 4, enhanced performance of *P. xylostella* and its parasitoid *D. semiclausum* was recorded when the caterpillars were feeding on aphid-infested plants. In contrast, aphid infestation did not affect the performance of *M. brassicae* nor its parasitoid *M. mediator*, which suggests species-specific effects of aphid infestation on plant direct defences against caterpillars. Plant phenotypic changes generally affect the hosts and their larval endoparasitoids in the same direction (Gols and Harvey, 2009). Increased performance of caterpillars on aphid-infested plant may result from SA-induced suppression of JA-responsive defences. Alternatively, aphid infestation may increase the nutritional quality of the plants for *P. xylostella* (Sandstrom et al., 2000) but not for *M. brassicae* caterpillars.

Previous studies have also reported that aphid infestation can promote the development of chewing herbivores (Rodriguez-Saona et al., 2005; Soler et al., 2012), but the underlying mechanisms involving phytohormones and transcription factors are rarely studied (Kroes et al., 2015). Future studies should aim to elucidate these at different levels, from the regulation of plant signalling pathways to changes in plant nutritional quality, *e.g.* production of anti-digestive compounds, toxins or primary metabolites. In brassicaceous plants, the effects of glucosinolates on the development of aphids and caterpillars are well studied, *e.g.* reproduction of *M. persicae* and *B. brassicae* aphids was negatively correlated with both indole and aliphatic glucosinolate content in Arabidopsis (De Vos et al., 2007); the

performance of caterpillars of *P. rapae* and *M. brassicae* correlated with concentrations of specific glucosinolates in the leaves of the various cabbage populations (Gols et al., 2008). Another secondary metabolite, kaempferol-3,7-dirhamnoside (KRR) in Arabidopsis, was shown to function as a defensive metabolite against *P. brassicae* caterpillars (Onkokesung et al., 2014). However, how dual infestation with both aphids and caterpillars influences plant production of secondary metabolites such as glucosinolates, remains largely unexplored. The investigation of plant non-volatile chemistry in relation to insect development will help us to understand the mechanisms underlying the observed effects of aphid infestation on the performance of the caterpillars and their parasitoids.

In Chapter 5, I investigated how aphid infestation affects plant indirect defences against caterpillars, *i.e.* foraging behaviour of parasitoids mediated by plant volatile emission. Previous studies have shown that aphid infestation induced plant volatiles that attract natural enemies of aphids (De Vos et al., 2007; Guerrieri and Digilio, 2008), but less is known about how aphid-induced changes in plant volatile emission may affect the plant's indirect defences against chewing herbivores that feed on the same host plant. Phloem-feeding whiteflies (*Bemisia tabaci*) negatively affected indirect plant defences against both *P. xylostella* caterpillars and *T. urticae* spider mites (Zhang et al., 2009; Zhang et al., 2013). Another study showed that foraging behaviour of *Cotesia glomerata*, a parasitoid of *P. brassicae*, was affected by the simultaneous presence of aphids (*B. brassicae*) and host caterpillars in an aphid density-dependent manner (Ponzio et al., 2016). Data presented in this thesis show that the effects of aphid infestation on plant indirect defences against caterpillars are highly species-specific and also depend on the period of aphid infestation. For instance, plants infested with aphids for a short period followed by caterpillar infestation were more attractive to *D. semiclausum* than plants infested with only host caterpillars. However, when the aphid infestation period was extended, plants infested with hosts alone were more attractive to *D. semiclausum* than the dually-infested plants. The reduced emission of HIPV by plants exposed to hosts plus extended aphid infestation could explain the reduced attraction of *D. semiclausum* to volatiles emitted by these plants. Interestingly, aphid infestation also reduced the volatile emission rate of plants infested with *M. brassicae*, but plants with dual infestation of both aphids and caterpillars were even more attractive to the parasitoid *M. mediator*. In addition to volatile emission, alternative plant traits, such as availability of food source (*e.g.* aphid honeydew) may also play a role in the attraction of parasitoids (Stapel et al., 1997; Amat et al., 2012).

Aphid infestation interfered with plant direct defence against leaf-chewing herbivores, depending on the identity of the herbivore. Moreover, the performance and the attraction of parastioids to volatiles was sometimes affected in opposite directions by co-infestation with aphids, depending on density of the aphids. Therefore, we could not provide a clear picture of how aphid infestation affected plant indirect defences against leaf-chewing herbivores. Nevertheless, aphid infestation altered plant direct and indirect defences

against leaf-chewers, and may potentially affect colonisation of host plants by other members of the associated community and, consequently, community assembly and structure (Ode, 2006; Dicke et al., 2009; Stam et al., 2014). Not only natural enemies of herbivores, but also herbivores themselves use plant-based infochemicals for host location (Bruce et al., 2005; Bruce and Pickett, 2011; Meiners, 2015).

Data presented in Chapter 3 show that early-season aphid herbivory only affected the abundance of a subset of the community, *i.e.* the natural enemies of aphids, but did not affect the chewing herbivores. The natural enemies of aphids may better perceive and respond to the volatiles induced by their hosts / prey. Butterflies and moths may not respond to the aphid-altered plant traits in terms of oviposition behaviour. Thus, these herbivores may be equally attracted to plants with or without early-season aphid infestation. Compared to the community-wide effects of early caterpillar infestation (Van Zandt and Agrawal, 2004; Viswanathan et al., 2005; Poelman et al., 2008; Poelman et al., 2010), the effects of early-season aphid infestation on a subset of the insect community imply a limited effect size of aphid-altered plant phenotypic changes. Moreover, the effect of aphid infestation was only significant in the first half of the season (June and July), but waned in the second half of the season (August and September). The negative effects of aphid infestation on induced plant defences against leaf-chewing herbivores may be transient, and only statistically significant in the lab, but not ecologically significant in the field over longer time spans. Alternatively, plants may adjust their response when attacked by other herbivores arriving later in the season, and the effect of early-season aphid infestation can then be diluted. To examine the long-term effects of aphid infestation on other herbivores occurring on the same host in the field, it is crucial to verify the results of individual species in a field study including all community members at different trophic levels.

## Intraspecific Variation In Plant Defence

Within plant species, there is natural variation in development and physiology among populations and individual (Alonso-Blanco et al., 2009). The variation in plant traits among individuals collected from different geographical regions is presumed to be ecologically important so that the plant is adapted to the local environment, and this may provide the basis for genetic variation (Alonso-Blanco and Koornneef, 2000). Many studies have reported significant levels of genetic variation in both constitutive and induced plant defences among plant genotypes and their effects on population growth of herbivores (Underwood and Rausher, 2000; Agrawal et al., 2002; Gols et al., 2008; Agrawal et al., 2012). To investigate whether the induced plant responses are conserved in natural plants, it is essential to study induced plant traits in different plant populations and genotypes when attacked by different herbivores (Johnson and Agrawal, 2007; Poelman et al., 2009).

In the model plant Arabidopsis, structural diversity in glucosinolate profiles is determined by polymorphism at five genetic loci which allows for the rapid generation of new glucosinolate combinations in response to heterogeneous natural selection (Alonso-Blanco and Koornneef, 2000). In the leaves of 39 Arabidopsis ecotypes from diverse geographical and environmental ranges, a 20-fold difference in the total concentration of aliphatic glucosinolates was found (Kliebenstein et al., 2001). Although not geographically distant, the three wild cabbage populations used in this study (KIM, OH and WIN) also exhibit variation in secondary chemistry, such as aliphatic glucosinolates, which was shown to correlate with differences in the abundance of the aphid *B. brassicae* and the micromoth *Selania leplastriana* in the field (Moyes et al., 2000; Newton et al., 2009). These populations also differ in the emission of volatile secondary metabolites when challenged by *P. rapae* feeding (Gols et al., 2011). Herbivore-induced levels of both volatile and non-volatile secondary metabolites in KIM plants differ from levels found in OH and WIN plants (Gols et al., 2008; Gols et al., 2011).

### Plant perspective

In this thesis project, I assessed the level of intraspecific variation in herbivore-induced volatile chemistry among the three cabbage populations. When the volatile blends emitted by host-infested plants and dually-infested plants with both aphids and hosts were compared, the overall pattern was similar across the three cabbage populations, *i.e*. total blend composition was similar in host-only and dually-infested plants (Chapter 5). However, there was variation among the cabbage populations when we compared individual volatile compounds emitted by plants exposed to the two herbivore treatments. In plants infested with *P. xylostella,* reduced emission rates in response to extended aphid feeding were only recorded in OH and WIN plants but not in KIM plants (Chapter 5).

To study the molecular mechanism underlying the induction of plant defences, we compared transcript levels of marker genes of the two major plant signalling pathways (Chapter 6). The overall transcription levels of *LOX* and *PR-1* varied among the three populations, but there was no interaction between aphid and caterpillar infestation on the transcript levels of these two genes and this pattern was consistent across the cabbage populations (Chapter 6).



### Insect perspective

I indirectly tested how aphid-induced plant responses differ among the cabbage populations by measuring performance of caterpillars and their parasitoids and preference behaviour of parasitoids. I recorded differential performance of *P. xylostella* and *M. brassicae* on aphid-infested plants of the three cabbage populations. Among the three cabbage populations,

*P. xylostella* developed the fastest on KIM plants, and *M. brassicae* gained the lowest biomass on WIN plants (Chapter 4). There are population-specific effects with respect to direct defences, such as constitutive and inducible chemical defences (Gols et al., 2008). However, preference behaviour of both parasitoids (*D. semiclausum* and *M. mediator*) for volatiles from plants infested with hosts plus aphids versus plants infested with aphids alone was consistent across the cabbage populations (Chapter 5). The intraspecific variation in defence traits among the three wild cabbage populations seems more pronounced with respect to plant direct than to indirect defences, as the performance of caterpillars but not the preference of parasitoids varied among the cabbage populations (Chapter 4 & 5). In Chapter 3, we investigated the effects of early-season aphid infestation on the insect community associated with the wild cabbage populations. Chapter 3 shows that the effect size of aphid infestation on insect community composition differed among the cabbage populations.

## Species-specificity in induced plant responses

Plants have evolved specific responses to cope with attack by various herbivorous insects with different feeding strategies and life styles. Feeding guild and the level of food-plant specialization of the herbivore are important factors that can determine the nature of the induced plant response (Voelckel and Baldwin, 2004; Bidart-Bouzat and Kliebenstein, 2011; Ali and Agrawal, 2012). Herbivore species-specific responses of plants were even found in response to infestation with various whitefly species and even to different developing stages of whiteflies (Walling, 2000).

The specific plant responses induced by herbivores may lead to changes in plant quality that affect the performance and behaviour of subsequent herbivores differently (Agrawal, 1998; Agrawal, 2000; Kessler and Baldwin, 2004; Poelman et al., 2008). Apart from the inducing herbivores, other responding herbivore species, as well as predators and parasitoids that are associated with the same plants, in turn, may also show variation in their responses to induced plant responses. Insect herbivores with different feeding strategies might differ in how they are affected by changes in plant traits. For example, stem-boring species may be more affected by changes in plant architecture and organ enlargement, whereas leaf-feeding and sap-feeding herbivores may be more affected by changes in nutrient quality (Chen and Bernal, 2011; Chen et al., 2015). Some studies have demonstrated that induced plant responses can attract some herbivore species that are searching for oviposition sites while repelling others (Agrawal, 2000; Kessler and Baldwin, 2004; Poelman et al., 2008).

Aphid feeding can result in species-specific responses of other herbivores that feed on the same plant individual (Chapter 4). We found that aphid infestation of plants enhanced the performance of the specialist caterpillar *P. xylostella* and its parastioid *D. semiclausum*, but

did not affect the performance of the generalist caterpillar *M. brassicae* or its parasitoid *M. mediator*. Previous studies have reported both positive and negative interactions between phloem-sucking and leaf-chewing caterpillars when they are feeding on the same plant. Performance and survival of the leaf-chewing cabbage looper *Trichoplusia ni* was negatively affected by infestation of the phloem-feeding whitefly *Bemisia argentifolii* (Inbar et al., 1999). A positive interaction between leaf-rolling caterpillars and aphids was observed, resulting from the construction of leaf shelters (Ohgushi, 2008). In Y-tube olfactometer experiments (Chapter 5), we found that aphid infestation both positively and negatively affected the foraging behaviour of a parasitoid depending on the duration of aphid infestation (Chapter 5). In addition, this effect was only found for one of the tested parasitoid species (*D. semiclausum*), whereas the behaviour of the other parasitoid (*M. mediator*) was positively affected by aphids irrespective of aphid-infestation duration. The results of this thesis demonstrate that insect species can respond differentially, sometimes contrastingly, to plant responses induced by the same herbivore species.

## Trade-off between plant direct and indirect defences

Plants are predicted to benefit from induced responses by reducing the survival and / or performance of herbivores (direct defences), and/or increasing the attraction and activity of natural enemies of herbivores (indirect defences). The induction of direct as well as indirect plant defensive traits in response to herbivory are regulated by the same intricate networks of signal-transduction pathways (Thaler et al., 2012). Plant physical constraints and resource limitation may result in trade-offs between direct and indirect defences. For example, the production of toxic hydrogen cyanide, a direct defence trait, and the release of plant volatiles, an indirect defence trait, were negatively correlated in Lima bean plants (Ballhorn et al., 2008). Moreover, the distinction between plant direct and indirect defences is not definite, *i.e.* direct defences may affect the behaviour and performance of the herbivore's natural enemies and indirect defences also affect the natural enemy's prey or hosts (Gols, 2014). For instance, some compounds serve as both direct and indirect defensive traits, e.g. linalool (Kessler and Baldwin, 2001) and isothiocyanate (Bradburne and Mithen, 2000). In nature, plants are often attacked my several herbivore species and trade-offs between direct and indirect plant defences may compromise a plant's ability to combine different traits for the achievement of optimal protection (Ballhorn et al., 2008). Therefore, plants may need to switch between direct and indirect defences depending on specific biotic stresses in time. In our experiments, aphid seemed to suppress direct plant defences initially but enhanced indirect defences against *P. xylostella*: performance of *P. xylostella* was better on plants co-infested with aphids (Chapter 4), and the parasitoid *D. semiclausum* was attracted to the dually infested plants over plants infested with only hosts (Chapter 5). Kroes *et al.* (2015) reported that positive effects of aphid infestation on

the performance of *P. xylostella* caterpillar were transient, and negative effects were found at a higher aphid density. Interestingly, *D. semiclausum* showed a reversed preference to the host-infested plants at higher aphid density (Chapter 5). A positive correlation between preference behaviour and performance of parasitoids has been reported previously (Gols et al., 2009; Kos et al., 2012). In this thesis, we also found that the parasitoid *D. semiclausum* preferred volatiles from plants that benefit their performance.

## Concluding remarks

The research presented in this thesis demonstrates that aphid infestation interfered with plant direct and indirect defences against leaf-chewing caterpillars but whether this occurred or not depended on the specific herbivore-parasitoid interaction and the duration of aphid infestation. Moreover, there is plant-population-specific variation in the effects of aphid infestation on plant direct defences against caterpillars and only small effects on caterpillar-induced volatile emission, but not on the behaviour of parasitoids. Comparison of transcripts of an SA- and JA-marker gene in plants with dual and single infestation did not provide support for SA-JA antagonism at the molecular level. Specificity of elicitation, *i.e.* the differential response of plants to attack by different herbivores and specificity of the effect, i.e. the differential response of insects associated to a plant with a given phenotype, can shape arthropod community structure (Ohgushi, 2005; Utsumi et al., 2010; Utsumi et al., 2010; Stam et al., 2014). Aphid infestation in this study only affected a subset of the plant-associated insect community, i.e. the abundance of natural enemies of aphids but not that of leaf-chewing herbivores or their natural enemies. Compared to the interactions between individual species, the influence of induction on the entire food web is much more complex, and the effects may change over time and space. The majority of the studies on tritrophic plant-insect interactions focus on interactions between herbivores and their natural enemies with single species at each trophic level. More studies investigating the effects of induced responses to herbivory on arthropod community structure are required (Agrawal, 1999; Poelman et al., 2008; Poelman et al., 2010; Agrawal et al., 2014). To draw general conclusions of how induced plant responses may be conserved in plants, we need to expand the research from a single genotype to multiple individual and / or plant populations (Poelman et al., 2009). There may be intraspecific variation in inducible plant traits resulting from the interaction with local insect communities, whereas consistency of induced plant responses among plant populations could provide solid evidence for conserved plant phenotypic changes.

## References


Agrawal A, Hastings A, Patrick E, Knight A (2014) Specificity of herbivore-induced hormonal signaling and defensive traits in five closely related milkweeds (*Asclepias* spp.). Journal of Chemical Ecology 40: 717-729.

Agrawal AA (1998) Induced responses to herbivory and increased plant performance. Science 279: 1201-1202.

Agrawal AA (1999) Induced responses to herbivory in wild radish: effects on several herbivores and plant fitness. Ecology 80: 1713-1723.

Agrawal AA (2000) Specificity of induced resistance in wild radish: causes and consequences for two specialist and two generalist caterpillars. Oikos 89: 493-500.

Agrawal AA, Conner JK, Johnson MT, Wallsgrove R (2002) Ecological genetics of an induced plant defense against herbivores: additive genetic variance and costs of phenotypic plasticity. Evolution 56: 2206-2213.

Agrawal AA, Hastings AP, Johnson MT, Maron JL, Salminen J-P (2012) Insect herbivores drive real-time ecological and evolutionary change in plant populations. Science 338: 113-116.

Agrawal AA, Karban R (1999) Why induced defenses may be favored over constitutive strategies in plants. In R Tollrian and CD Harvell (Eds.) *The ecology and evolution of inducible defenses* (pp. 45-61). Princeton, US: Princeton University Press.

Ali JG, Agrawal AA (2012) Specialist versus generalist insect herbivores and plant defense. Trends in Plant Science 17: 293-302.

Alonso-Blanco C, Aarts MG, Bentsink L, Keurentjes JJ, Reymond M, Vreugdenhil D, Koornneef M (2009) What has natural variation taught us about plant development, physiology, and adaptation? The Plant Cell 21: 1877-1896.

Alonso-Blanco C, Koornneef M (2000) Naturally occurring variation in *Arabidopsis*: an underexploited resource for plant genetics. Trends in Plant Science 5: 22-29.

Amat I, Besnard S, Foray V, Pelosse P, Bernstein C, Desouhant E (2012) Fuelling flight in a parasitic wasp: which energetic substrate to use? Ecological Entomology 37: 480-489.

Ballhorn DJ, Kautz S, Lion U, Heil M (2008) Trade-offs between direct and indirect defences of lima bean (*Phaseolus lunatus*). Journal of Ecology 96: 971-980.

Beckers GJM, Spoel SH (2006) Fine-tuning plant defence signalling: salicylate versus jasmonate. Plant Biology 8: 1-10.

Bidart-Bouzat MG, Kliebenstein D (2011) An ecological genomic approach challenging the paradigm of differential plant responses to specialist versus generalist insect herbivores. Oecologia 167: 677-689.

Bradburne RP, Mithen R (2000) Glucosinolate genetics and the attraction of the aphid parasitoid *Diaeretiella rapae* to Brassica. Proceedings of the Royal Society of London B: Biological Sciences 267: 89-95.

Briggs D, Walters SM (1997) Plant variation and evolution. Cambridge University Press

Bruce TJ, Wadhams LJ, Woodcock CM (2005) Insect host location: a volatile situation. Trends in Plant Science 10: 269-274.

Bruce TJA, Pickett JA (2011) Perception of plant volatile blends by herbivorous insects - Finding the right mix. Phytochemistry 72: 1605-1611.

Chen YH, Bernal CC (2011) Arthropod diversity and community composition on wild and cultivated rice. Agricultural and Forest Entomology 13: 181-189.

Chen YH, Gols R, Benrey B (2015) Crop domestication and its impact on naturally selected trophic interactions. Annual Review of Entomology 60: 35-58.

De Vos M, Kim JH, Jander G (2007) Biochemistry and molecular biology of Arabidopsis-aphid interactions. BioEssays 29: 871-883.

Dicke M, Baldwin IT (2010) The evolutionary context for herbivore-induced plant volatiles: beyond the 'cry for help'. Trends in Plant Science 15: 167-175.

Dicke M, Van Loon JJA, Soler R (2009) Chemical complexity of volatiles from plants induced by multiple attack. Nature Chemical Biology 5: 317-324.

Engelberth J, Koch T, Schüler G, Bachmann N, Rechtenbach J, Boland W (2001) Ion channel-forming alamethicin is a potent elicitor of volatile biosynthesis and tendril coiling. Cross talk between jasmonate and salicylate signaling in lima bean. Plant Physiology 125: 369-377.

Erb M, Meldau S, Howe GA (2012) Role of phytohormones in insect-specific plant reactions. Trends in Plant Science 17: 250-259.

Gols R (2014) Direct and indirect chemical defences against insects in a multitrophic framework. Plant, Cell and Environment 37: 1741-1752.

Gols R, Bullock JM, Dicke M, Bukovinszky T, Harvey JA (2011) Smelling the wood from the trees: non-linear parasitoid responses to volatile attractants produced by wild and cultivated cabbage. Journal of Chemical Ecology 37: 795-807.

Gols R, Harvey JA (2009) Plant-mediated effects in the Brassicaceae on the performance and behaviour of parasitoids. Phytochemistry Reviews 8: 187-206.

Gols R, van Dam NM, Raaijmakers CE, Dicke M, Harvey JA (2009) Are population differences in plant quality reflected in the preference and performance of two endoparasitoid wasps? Oikos 118: 733-743.

Gols R, Wagenaar R, Bukovinszky T, van Dam NM, Dicke M, Bullock JM, Harvey JA (2008) Genetic variation in defense chemistry in wild cabbages affects herbivores and their endoparasitoids. Ecology 89: 1616-1626.

Guerrieri E, Digilio MC (2008) Aphid-plant interactions: a review. Journal of Plant Interactions 3: 223-232.

Hare JD (2011) Ecological role of volatiles produced by plants in response to damage by herbivorous insects. Annual Review of Entomology 56: 161-180.

Heil M (2008) Indirect defence via tritrophic interactions. New Phytologist 178: 41-61

Howe GA, Jander G (2008) Plant immunity to insect herbivores. Annual Review of Plant Biology 59: 41-66.

Inbar M, Doostdar H, Mayer RT (1999) Effects of sessile whitefly nymphs (Homoptera: Aleyrodidae) on leaf-chewing larvae (Lepidoptera: Noctuidae). Environmental Entomology 28: 353-357.

Johnson MTJ, Agrawal AA (2007) Covariation and composition of arthropod species across plant genotypes of evening primrose, *Oenothera biennis*. Oikos 116: 941-956.

Karban R, Baldwin I (1997) Induced responses to herbivory University of Chicago Press. Chicago, Illinois, USA.

Kessler A, Baldwin IT (2001) Defensive function of herbivore-induced plant volatile emissions in nature. Science 291: 2141-2144.

Kessler A, Baldwin IT (2002) Plant responses to insect herbivory: the emerging molecular analysis. Annual Review of Plant Biology 53: 299-328.

Kessler A, Baldwin IT (2004) Herbivore-induced plant vaccination. Part I. The orchestration of plant defenses in nature and their fitness consequences in the wild tobacco *Nicotiana attenuata*. The Plant Journal 38: 639-649.

Kessler A, Halitschke R (2007) Specificity and complexity: the impact of herbivore-induced plant responses on arthropod community structure. Current Opinion in Plant Biology 10: 409-414.

Kliebenstein DJ, Kroymann J, Brown P, Figuth A, Pedersen D, Gershenzon J, Mitchell-Olds T (2001) Genetic control of natural variation in Arabidopsis glucosinolate accumulation. Plant physiology 126: 811-825.

Koornneef A, Pieterse CMJ (2008) Cross talk in defense signaling. Plant Physiology 146: 839-844.

Kos M, Houshyani B, Achhami BB, Wietsma R, Gols R, Weldegergis BT, Kabouw P, Bouwmeester HJ, Vet LEM, Dicke M, van Loon JJA (2012) Herbivore-mediated effects of glucosinolates on different natural enemies of a specialist aphid. Journal of Chemical Ecology 38: 100-115.

Kroes A, van Loon JJA, Dicke M (2015) Density-dependent interference of aphids with caterpillar-induced defenses in Arabidopsis: involvement of phytohormones and transcription factors. Plant and Cell Physiology 0: 1-9.

Meiners T (2015) Chemical ecology and evolution of plant–insect interactions: a multitrophic perspective. Current Opinion in Insect Science 8: 22-28.

Moyes CL, Collin HA, Britton G, Raybould AE (2000) Glucosinolates and differential herbivory in wild populations of *Brassica oleracea*. Journal of Chemical Ecology 26: 2625-2641.

Moyes CL, Raybould AF (2001) The role of spatial scale and intraspecific variation in secondary chemistry in host-plant location by *Ceutorhynchus assimilis* (Coleoptera : Curculionidae). Proceedings of the Royal Society B: Biological Sciences 268: 1567-1573.

Newton EL, Bullock JM, Hodgson D (2009) Bottom-up effects of glucosinolate variation on aphid colony dynamics in wild cabbage populations. Ecological Entomology 34: 614-623.

Ode PJ (2006) Plant chemistry and natural enemy fitness: effects on herbivore and natural enemy interactions. Annual Review of Entomology 51: 163-185.

Ohgushi T (2005) Indirect interaction webs: herbivore-induced effects through trait change in plants. Annual Review of Ecology, Evolution, and Systematics 36: 81-105.

Ohgushi T (2008) Herbivore-induced indirect interaction webs on terrestrial plants: the importance of non-trophic, indirect, and facilitative interactions. Entomologia Experimentalis et Applicata 128: 217-229.

Onkokesung N, Reichelt M, van Doorn A, Schuurink RC, van Loon JJA, Dicke M (2014) Modulation of flavonoid metabolites in *Arabidopsis thaliana* through overexpression of the MYB75 transcription factor: role of kaempferol-3,7-dirhamnoside in resistance to the specialist insect herbivore *Pieris brassicae*. Journal of Experimental Botany 65: 2203-2217.

Ozawa R, Arimura G-i, Takabayashi J, Shimoda T, Nishioka T (2000) Involvement of jasmonate- and salicylate-related signaling pathways for the production of specific herbivore-induced volatiles in plants. Plant and Cell Physiology 41: 391-398.

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal modulation of plant immunity. Annual Review of Cell and Developmental Biology 28: 28.21-28.33.

Poelman EH, Broekgaarden C, Van Loon JJA, 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 EH, Dam NM, Loon JJA, Vet LEM, Dicke M (2009) Chemical diversity in *Brassica oleracea* affects biodiversity of insect herbivores. Ecology 90: 1863-1877.

Poelman EH, Van Loon JJA, Van Dam NM, Vet LEM, Dicke M (2010) Herbivore-induced plant responses in *Brassica oleracea* prevail over effects of constitutive resistance and result in enhanced herbivore attack. Ecological Entomology 35: 240-247.

Ponzio C, Cascone P, Cusumano A, Weldegergis BT, Fatouros NE, Guerrieri E, Dicke M, Gols R (2016). Volatile-mediated foraging behaviour of three parasitoid species under conditions of dual insect herbivore attack. Animal Behaviour 111: 197-206.

Rapusas HR, Bottrell DG, Coll M (1996) Intraspecific variation in chemical attraction of rice to insect predators. Biological Control 6: 394-400.

Rodriguez-Saona C, Chalmers JA, Raj S, Thaler JS (2005) Induced plant responses to multiple damagers: differential effects on an herbivore and its parasitoid. Oecologia 143: 566-577.

Sandstrom J, Telang A, Moran NA (2000) Nutritional enhancement of host plants by aphids - a comparison of three aphid species on grasses. Journal of Insect Physiology 46: 33-40.

Schoonhoven LM, van Loon JJA, Dicke M (2005) Insect-Plant Biology, 2nd edn. Oxford University Press, Oxford.

Soler R, Badenes-Perez FR, Broekgaarden C, Zheng SJ, David A, Boland W, Dicke M (2012) Plant-mediated facilitation between a leaf-feeding and a phloem-feeding insect in a brassicaceous plant: from insect performance to gene transcription. Functional Ecology 26: 156-166.

Stam JM, Kroes A, Li Y, Gols R, van Loon JJA, Poelman EH, Dicke M (2014) Plant interactions with multiple insect herbivores: from community to genes. Annual Review of Plant Biology 65: 689-713.

Stapel JO, Cortesero AM, De Moraes CM, Tumlinson JH, Lewis WJ (1997) Extrafloral nectar, honeydew, and sucrose effects on searching behavior and efficiency of *Microplitis croceipes* (Hymenoptera: Braconidae) in cotton. Environmental Entomology 26: 617-623.

Thaler JS, Humphrey PT, Whiteman NK (2012) Evolution of jasmonate and salicylate signal crosstalk. Trends in Plant Science 17: 260-270.

Turlings TCJ, Tumlinson JH, Lewis WJ (1990) Exploitation of her.bivore-induced plant odors by host-seeking parasitic wasps. Science 250: 1251-1253

Underwood N, Rausher MD (2000) The effects of host-plant genotype on herbivore population dynamics. Ecology 81: 1565-1576.

Utsumi S, Ando Y, Miki T (2010) Linkages among trait-mediated indirect effects: a new framework for the indirect interaction web. Population Ecology 52: 485-497.

Utsumi S, Kishida O, Ohgushi T (2010) Trait-mediated indirect interactions in ecological communities. Population Ecology 52: 457-459.

Utsumi S, Ohgushi T (2008) Host plant variation in plant-mediated indirect effects: moth boring-induced susceptibility of willows to a specialist leaf beetle. Ecological Entomology 33: 250-260.

van Poecke RMP, Dicke M (2002) Induced parasitoid attraction by *Arabidopsis thaliana*: involvement of the octadecanoid and the salicylic acid pathway. Journal of Experimental Botany 53: 1793-1799.

Van Zandt PA, Agrawal AA (2004) Community-wide impacts of herbivore-induced plant reaponses in milkweed (*Asclepias syriaca*). Ecology 85: 2616-2629.

Vet LEM, Dicke M (1992) Ecology of infochemical use by natural enemies in a tritrophic context. Annual Review of Entomology 37: 141-172.

Viswanathan DV, Narwani AJT, Thaler JS (2005) Specificity in induced plant responses shapes patterns of herbivore occurrence on *Solanum dulcamara*. Ecology 86: 886-896.

Voelckel C, Baldwin IT (2004) Generalist and specialist lepidopteran larvae elicit different transcriptional responses in *Nicotiana attenuata*, which correlate with larval FAC profiles. Ecology Letters 7: 770-775.

Voelckel C, Baldwin IT (2004) Herbivore-induced plant vaccination. Part II. Array-studies reveal the transience of herbivore-specific transcriptional imprints and a distinct imprint from stress combinations. The Plant Journal 38: 650-663.

Walling LL (2000) The myriad plant responses to herbivores. Journal of Plant Growth Regulation 19: 195-216.

Zhang P, Broekgaarden C, Zheng S, Snoeren TAL, Loon JJAv, Gols R, Dicke M (2013) Jasmonate and ethylene signaling mediate whitefly-induced interference with indirect plant defense in *Arabidopsis thaliana*. New Phytologist 197: 1291-1299.

Zhang PJ, Li WD, Huang F, Zhang JM, Xu FC, Lu YB (2013) Feeding by whiteflies suppresses downstream jasmonic acid signaling by eliciting salicylic acid signaling. Journal of Chemical Ecology 39: 612-619.

Zhang PJ, Zheng SJ, van Loon JJA, Boland W, David A, Mumm R, Dicke M (2009) Whiteflies interfere with indirect plant defense against spider mites in Lima bean. Proceedings of the National Academy of Sciences of the United States of America 106: 21202-21207.

Zheng S-J, Dicke M (2008) Ecological genomics of plant-insect interactions: from gene to community. Plant Physiology 146: 812-817.

## Summary

As primary producers in terrestrial ecosystems, plants are often attacked by a variety of herbivorous insects. Herbivorous insects are the most diverse group among herbivorous animals, and have a long evolutionary history with their host plants. To cope with the diversity of herbivorous attackers, plants have developed direct and indirect defences, including chemical, physiological and morphological traits that prevent or reduce the survival and/or growth of the herbivores and promote the activity of the natural enemies of insect herbivores. Upon herbivore attack, plants may respond with phenotypic changes that do not only function as defensive mechanisms against the inducing attackers, but may also affect other herbivores that occur on the same host plant simultaneously or subsequently. Moreover, the herbivore-induced plant phenotypic changes may further influence the structure and dynamics of the associated insect community.

Herbivore-induced plant responses are often species-specific. Feeding guild and dietary specialization of the inducing herbivores are important factors determining the specificity of plant responses. For instance, phloem-feeding herbivores predominantly induce salicylic acid (SA) dependent plant defences, whereas leaf-chewing herbivores mainly induce jasmonic acid (JA) dependent defences. Furthermore, crosstalk between SA and JA signaling pathways may help plants to fine-tune their defences against both phloem-feeding and leaf-chewing herbivores, but may also constrain a plant's ability to defend itself against both types of herbivores simultaneously. Using wild cabbage (*Brassica oleracea*) populations, this thesis explores whether aphid-infestation interferes with the response to chewing herbivores and whether this impacts performance and behaviour of individual chewing insect herbivores and their natural enemies, as well as the entire insect community. I investigated this using three wild cabbage populations that are known to differ in inducible secondary chemistry, to reveal whether patterns were consistent.

A literature review on recent developments in the field of plant interactions with multiple herbivores (Chapter 2) addressed how plant traits mediate interactions with various species of the associated insect community and their dynamics. In addition, the mechanisms underlying phenotypic changes in response to different herbivores were discussed from the expression of defence-related genes, phytohormones and secondary metabolites in plants to their effects on the performance and behaviour of individual insects as well as the entire insect community. Previous studies mainly focused on multi-trophic interactions between plants, herbivores, parasitoids / predators with single species at each trophic level. To understand phenotypic changes in response to herbivory and their effects on associated insect community members, it is important to take a multidisciplinary approach that investigates and integrates the effects of multiple attack on plants at different levels of biological organization.

Early-season herbivory by chewing insects has been reported to have community-wide effects throughout the season, but little is known about the effects of phloem-feeding herbivores on

the structure and dynamics of the insect community. In Chapter 3, I investigated the effects of early-season infestation by the aphid *Brevicoryne brassicae* on the composition and dynamics of the entire insect community throughout the season in a garden experiment replicated in two consecutive years. Aphid infestation in the early season only affected a subset of the community, *i.e.* the natural enemies of aphids, but not the chewing herbivores and their natural enemies. Moreover, the effects were only significant in the first half (June & July), but waned in the second half of the season (August & September). The effect of aphid infestation on the community of natural enemies also varied among the cabbage populations. Results of this study and those reported for the effects of early season herbivory by chewing insects suggest that plant phenotypic changes induced by early-season herbivory and plant genotypic variation can affect the abundance of other community members, but the identity of the inducing herbivore may determine the effect size and which community members may be affected.

In long-term experiments encompassing the whole season, plants may adjust their responses when attacked by other herbivores arriving later in the season, and as a result of this the effect of early-season aphid induction may be transient as I showed in Chapter 3. Chapter 4 investigated the effects of aphid infestation on plant direct defences against chewing herbivores in short-term laboratory experiments by comparing the performance of chewing herbivores and their parasitoids on aphid-infested and aphid-free plants. The performance of the specialist herbivore *Plutella xylostella* and its parasitoid *Diadegma semiclausum* was better on plants infested with aphids than on aphid-free plants, whereas the performance of the generalist herbivore *Mamestra brassicae* and its parasitoid *Microplitis mediator* was not affected by aphid infestation. These results suggest that aphid induced changes in plant traits may differentially affect the performance of leaf-chewing herbivore species attacking the same host plant. Thus, the effects of aphid infestation on plant direct defences against chewing herbivores are herbivore species-specific and they also varied among the cabbage populations.

Chapter 5 examined the short-term effects of *B. brassicae* aphid infestation on plant indirect defences against chewing herbivores. In a two-choice olfactometer bioassay, preference behaviour for volatiles emitted by plants infested with hosts alone and those emitted by plants infested with aphids and hosts was compared for *D. semiclausum* and *M mediator,* larval endoparasitoids of caterpillars of *P. xylostella* and *M. brassicae*, respectively. In addition, the headspace volatiles emitted by host-infested and dually-infested plants were collected and analyzed. Co-infestation with aphids differentially affected volatile-mediated foraging behaviour of the two parasitoid species in a density-dependent manner. *Diadegma semiclausum* preferred dually infested plants over host-infested plants when aphids infested the plants for a short time period, *i.e.* 7 days. However, volatile preference of *D. semiclausum* was reversed when aphid infestation was extended to 14 days. In contrast, *M. mediator* consistently preferred volatiles emitted by the dually-infested plants over those emitted by host-infested plants. The patterns of preference behaviour of the two wasp species were consistent across

the three cabbage populations. Interestingly, the emission rate of most volatile compounds was reduced in plants dually-infested with *M. brassicae* caterpillars and aphids compared to singly-infested with caterpillars. Volatile production by plants dually infested with aphids and *P. xylostella* was only reduced in plants exposed to aphids for two weeks in two of the three cabbage populations. This study showed that aphid infestation increased plant indirect defences against *M. brassicae, i.e.* it increased the attraction of *M. mediator*, but the effects on plant indirect defences against *D. semiclausum* depended on the aphid infestation period. Foraging behaviour of the parasitoids could not be fully explained by differences in chemical characteristics of the volatile blends. Other aphid-induced plant traits or the presence of the aphids themselves may be involved in the enhanced attraction of parasitoids of chewing herbivores to plants infested with both hosts and aphids.

We hypothesized a negative interference of aphid infestation on plant defences against chewing herbivores based on previously reported SA-JA antagonism. The antagonistic interaction between SA-JA signaling pathways has intensively been tested in the model plant Arabidopsis and other cultivated plants, but has rarely been studied in natural plants that have evolved defensive traits under conditions of natural selection. In Chapter 6, we assessed the activation of SA and JA signaling pathways in plants infested by both aphids (*B. brassicae*) and various caterpillar species (*P. xylostella, M. brassicae* and *Pieris brassicae*) in different time sequences by quantifying transcription levels of the SA- and JA-responsive marker genes, *PR-1* and *LOX* respectively. The results did not provide support for SA-JA antagonism. Compared to single infestation with each of the herbivore species, dual infestation with aphid and caterpillars had no interactive effects on the transcription levels of the SA- and JA-responsive maker genes, regardless of the temporal sequence of aphid and caterpillar attack, or the identity of the attacking caterpillar species. Aphid infestation may only induce limited SA production in wild *B. oleracea*, which could explain the observed non-interactive effects of dual infestation on plant activation of SA and JA signaling pathways. Alternatively, other genes may be involved in JA-SA antagonism in this species.

The findings of this thesis contribute to our understanding of plant responses to herbivory by insect species belonging to different feeding guilds and their ecological effects on other associated community members. Aphid infestation may interfere with plant direct and indirect defences against leaf-chewing herbivores at the individual species level, but the effects are species-specific and also depend on the infestation period of aphids. Early-season aphid infestation may further affect the composition of the insect community, but the effect is smaller influencing only a subset of the community compared to early infestation by chewing herbivores. The molecular mechanism underlying plant responses to both phloem-feeding and leaf-chewing herbivores are complex and require the investigation of a range of genes involved in JA- and SA-mediated defence signal transduction. Plant interact with multiple herbivores at different levels of biological organization ranging from the subcellular level to the individual and the community level, and an integrated multidisciplinary approach is required to investigate plant-insect interactions.

## Acknowledgements

Writing the acknowledgement brings back memories. When I look back at the past four years of my PhD, I am surprised about and grateful how inspiring the adventure to be trained as a scientist has been. I came to Wageningen for my MsC study, a long way from China, and was impressed by the research at Entomology during my MSc thesis work. After that, I was dedicated to pursue a PhD project and continue doing research here. I developed and matured dramatically in this period, not only technically mastering the scientific skills, but also mentally. Doing a PhD is intensive but also enjoyable, I have cried when experiments went into the wrong direction, but also I cannot be happier to see my publications online. I am proud to finish it, but with this thesis book in my hand, data and figures based on four-years of work I also feel a sense of being lost now this special period of my life is coming to an end. Doing a PhD is more like a unique experience of 'knowing yourself' rather than performing research, and it has helped me to find out what is my field of interest and a direction for a future career. Of course, I did not do this all on my own, I have always considered myself so lucky to get help from people during every stage of my life.

First of all, I like to express my special gratitude to my supervisors, Marcel and Rieta, I could not finished my PhD without your support and encouragement. Marcel, thank you for offering me the chance to work on your 'TOP project', I cannot forget how loudly I heralded when you announced that I was selected for this PhD position. You are such a great supervisor; you always make time for your students, no matter how busy you are. I received your revisions on my manuscripts late at night, during weekends and when you were abroad. You inspire all your students with your commitment to science, high standard for research and time management. On top of that, I really admire how you train the next generation of young scientists. Rieta, how lucky have I been to have you as my daily supervisor. Your supervision was indeed on a daily base in the past four years. I still remember that I phoned you when you were painting your house to ask questions about experiments. I cannot picture how you picked up the phone from the ladder. You were always available for discussions and questions, and even helping out in the intensive experimental days. You are amongst the very few researchers who stay close to practical work, and that's how you gain insights in the frontiers of Entomology, and never run out of new ideas.

I also need to give a big grateful thank to my fellow TOP-girls, Anneke and Jeltje. It was such a pleasure to be involved in this TOP project, and share all the inspiring discussions with you girls. Thank you for the brilliant collaboration, and being my close friends. Anneke, I appreciate all your patience in the molecular lab, and your cheerful words when I was 'down', I could not have completed the molecular chapter without your expertise. My son cannot fall asleep without your Nijntje, Anneke, I think this rabbit already has taken a place in his heart. Jeltje, we had a great time in the field, although working in adjacent fields, we were still visiting each other's field by bike, haha! You were curious why I was fully

covered from hair to toes even when working in the field, and you could not keep on talking about my answer that 'Asian girls do not appreciate tanning'. I always liked to tease you and Martine about how you 'guys' were glued together! How nice that I shared with you all my family stories, in the late evenings when only the two of us were writing in the office. Girls, I will regret if we will never meet each other again after I move back to China, so you should definitely visit me in China if you have the chance!

The Ento-family creates such a pleasant atmosphere with all the events, labuitjes, multicultural parties, YELREM, etc., that makes my memories of the time at Ento so colourful. A special thanks to all the people in the lab, my fellow PhD colleagues. Dani, we have known each other from the very beginning, thank you for all your support during my PhD and the movie nights with you. You made me feel I was never alone in this country. Nurmi, thank you for all the discussions about life, I enjoyed our shopping days at Utrecht, although I walk much faster than you, haha! Feng, you are the most positive guy I have ever met, you seem like having nothing to worry about in your entire life, I like your attitude for life, and this is something I should learn from you! Marjolein, we have become close friends in the last year of my PhD (why not earlier?! haha), you always know what you want, and you go directly for it, your visits during my maternity leave inspired me, yes, you are right, I should never give up my career for any reason! Karen, Cindy, Manus, Nelson, Alex, Shuhang, Keiko, Eddie, Camille, Tila, Jenny, Lucille, Kay, thanks for your kindness, and making this wonderful working environment! I also learned from the senior people in our lab, Nina, Patrick, Joop, Erik, thank you for sharing your knowledge and your patience in teaching young scientists! Ana and Roxina, you guided me into Entomology, and you showed me it is always possible to balance life and research. I cannot forget all the assistance and advice I got from you, both in professional and personal matters throughout my stay in Wageningen. Thanks to the rearing group of Entomology and Unifarm staff: none of the research could have been carried out without your assistance!

The students that I worked with deserve a big thanks! Surachet Chamontri, Wen Liu and Davy Meijer, you all worked hard on your projects, and contributed to this thesis book. Surachet, you managed to work with two species of parasitoids on all the treatments × population combinations which showed your passion for research. Wen Liu, I miss our girl's talk at the greenhouse of Unifarm, your sense of humour always delighted our busy experimental days. Davy, you had to re-schedule several times your experimental planning, and in the end all your efforts resulted in a good MSc thesis with a high grade. I am very proud to see you developed yourself during the thesis period at Ento, and I wish you all the best in your career!

我还要感谢我的家庭成员，黄科伟和黄蔚冶。我们仨就是一个家，人在家在，去哪里安身立命都不重要。我们每个人除了是对方最重要的家人，更是一个独立的个体，都有各自不同的追求和梦想，应该互相支持互相理解。我读博这四年来，走过

各种心路历程，感谢你黄科伟，分享我的点滴感受。虽然你不太会劝慰或者安抚我的小情绪，但是你是一个完美的倾听者，你常常是等让我自己去平复情绪，然后开始自我剖析，我觉得这其实就是最好的安慰。也感谢你常常在周末陪我来Radix加班，你的陪伴和耐心使我加班的那些周末都变成了甜蜜的回忆。尽管生活中我们有很多的见解不同，但是最终我们仍然选择给对方最大程度的包容和尊重，并且坚持走下去。我们一直在讨论和计划什么时候养育一个孩子，因为我们俩都那么爱小孩，敦敦的到来使我最后一年写论文的阶段变得尤其艰苦，在写论文遇到瓶颈时我曾经闪过一个念头，在博四论文阶段怀孕是不是不明智的，但是我又立马为自己有这样的想法感到愧疚和羞耻，敦敦值得最好的爱和无怨无悔的付出。敦敦的出生给了我很多的人生感悟，使我变得更宽容更平和也更有韧性了吧，是他在帮助我成长。我常常想为人父母应该在任何场合和时间都是平静中带着小喜悦的状态，就是各种突发事件都能妥善的不留痕迹的处理，所以我还需要变得更好，希望给敦敦树立一个好榜样吧。敦敦，你是一个那么爱笑的容易快乐的小男孩，是你让我感受到原来生活可以那么简单，原来幸福在于自己的心态。我常常都在感慨，命运是如此眷顾于我，让我可以与你结下生命中这段深厚的缘分，母子之缘。敦敦，你在妈妈肚子里的时候妈妈每天都在拼命写论文，你出生以后妈妈又忙于答辩发表和找职位，有时候真的疏于对你的照料，但是你是妈妈奋斗的动力，是妈妈对于生活全部热情的来源以及全部身心的寄托。

最后，我还要感谢我的父母，李田分和余琴娟，没有你们的支持，我不可能来到荷兰求学，没有你们的支持我也不可能如期提交博士论文，每一次跟你们的跨国长途通话都是我心灵的养分，是你们让我懂得了为人处事的道理和人生历练的意义所在。还有我的祖父母李树根和汤阿娣，是你们在农村对我从小的养育，激发了我对农业科学的兴趣，时令节气，农谚农歌是我最早的启蒙。我的祖父是我一生最敬重的人，是我第一个偶像，我永远记得在清晨的薄雾里，祖父，我还有大黄狗走在细长的田埂上，两旁是茄子，黄瓜，番茄生长拔节的声音。

Many many thanks to all of you, my dear family, supervisors, friends and colleagues, for being part of my life!

## Publication list

Soler R, Pineda A, Li Y, Ponzio C, van Loon JJA, Weldegergis BT, Dicke M (2012) Neonates know better than their mothers when selecting a host plant. Oikos 121 (12): 1923-1934.

Li Y, Dicke M, Harvey J, Gols R (2013) Intra-specific variation in wild *Brassica oleracea* for aphid-induced plant responses and consequences for caterpillar–parasitoid interactions. Oecologia 174 (3): 853-862.

Stam JM*, Kroes A*, Li Y*, Gols R, van Loon JJA, Poelman EH, Dicke M (2014) Plant Interactions with Multiple Insect Herbivores: From Community to Genes. Annual Review of Plant Biology 65: 689-713.

Li Y, Stam JM, Poelman EH, Dicke M and Gols R (2016) Community structure and abundance of insects in response to early-season aphid infestation in wild cabbage populations. Ecological Entomology (in press)

Li Y, Dicke M, Kroes A, Liu W and Gols R. Interactive effects of aphid and caterpillar herbivory on transcription of plant genes associated with phytohormonal signalling in wild cabbage. (accepted by Journal of Chemical Ecology)

Li Y, Weldegergis BT, Chamontri S, Dicke M and Gols R. Does aphid infestation interfere with plant indirect defence against lepidopteran caterpillars in wild cabbage? (under review by Oecologia)

Pineda A, Soler R, Pastor V, Li Y and Dicke M. Plant-mediated species networks: the modulating role of herbivore density. (submitted to Ecological Entomology)

## Curriculum vitae

Yehua Li was born on January 1st, 1986, at Liyang County in Jiangsu Province, China. She graduated at the high school of Liyang in 2003, and in the same year, she started her bachelor education in Plant Resource Utilization at Nanjing Forest University in Nanjing, China. In 2007, she enrolled in the Master program Plant Sciences at Wageningen University with a specialization in Plant Pathology and Entomology. She carried out her major and minor thesis on the topic of plant-mediated inter-guild interactions between phloem-feeding and leaf-chewing herbivores at the Laboratory of Entomology under the supervision of Dr. Roxina Soler and Dr. Ana Pineda. In 2011, she started her PhD project on Plant responses to multiple herbivory: phenotypic changes and their ecological consequences, at the Laboratory of Entomology of Wageningen Univerisity, under the supervision of promotor Prof. dr. Marcel Dicke and co-promotor Dr. Rieta Gols. In this thesis, the results obtained during her PhD are presented.

# Education Statement of the Graduate School Experimental Plant Sciences


**Issued to:** Yehua Li
**Date:** 1 September 2016
**Group:** Laboratory of Entomology
**University:** Wageningen University & Research

| 1) Start-up phase | *date* |
|---|---|
| ▶ **First presentation of your project** | |
| Ecological effects of phloem-feeding herbivore (Brevicoryne brassicae) on induced defences against a chewing herbivore in Brassica oleracea. | Mar 26, 2012 |
| ▶ **Writing or rewriting a project proposal** | |
| ▶ **Writing a review or book chapter** | |
| Plant interactions with multiple insect herbivores: from community to genes, Annu.Rev. Plant Biol. 2014, 65: 689-713. DOI: 10.1146/annurev-arplant-050213-035937 | Oct 2012-May 2013 |
| ▶ **MSc courses** | |
| Molecular Aspects of Bio-interactions (PHP30806) | Nov-Dec 2011 |
| ▶ **Laboratory use of isotopes** | |
| *Subtotal Start-up Phase* | *13.5 credits** |

| 2) Scientific Exposure | *date* |
|---|---|
| ▶ **EPS PhD student days** | |
| EPS PhD student day, University of Amsterdam | Nov 30, 2012 |
| EPS PhD student day, Leiden University | Nov 29, 2013 |
| ▶ **EPS theme symposia** | |
| EPS theme 2 'Interactions between Plants and Biotic Agents' and Willie Commelin Scholten Day, Utrecht University | Jan 24, 2013 |
| EPS theme 2 'Interactions between Plants and Biotic Agents' and Williie Commelin Scholten Day, University of Amsterdam | Feb 25, 2014 |
| EPS theme 3 'Metabolism and Adaptation', Wageninngen University | Mar 11, 2014 |
| ▶ **Lunteren days and other National Platforms** | |
| Netherlands Annual Ecology Meeting | Feb 05-06, 2013 |
| Netherlands Annual Ecology Meeting | Feb 11, 2014 |
| ▶ **Seminars (series), workshops and symposia** | |
| 6th workshop on Plant-Insect Interactions, University of Amsterdam | Nov 23, 2011 |
| Entomologendag 2011 | Dec 16, 2011 |
| Yearly Entomology Research Exchange Meeting | May 30, 2012 |
| 7th workshop on Plant-Insect Interactions, Leiden University | Nov 28, 2012 |
| Entomologendag 2012 | Dec 14, 2012 |
| Yearly Entomology Research Exchange Meeting May 17th 2013 | May 17, 2013 |
| 8th workshop on plant-insect interactions, Wageningen University | Sep 24, 2013 |
| Entomologendag 2013 | Dec 13, 2013 |
| 9th workshop on plant-insect interactions, Utrecht University | Nov 03, 2014 |
| WEES seminar: Koos Biesmeijer 'On bees, pollination and food security' | Dec 18, 2014 |

| | |
|---|---|
| Entomologendag 2014 | Dec 19, 2014 |
| WEES seminar: Kevin Foster 'The evolution of cooperation and competition in microbes' | Jan 22, 2015 |
| ▶ **Seminar plus** | |
| ▶ **International symposia and congresses** | |
| 14th International Symposium on Insect-Plant Relationships, Wageningen, The Netherlands | Aug 13-17, 2011 |
| 15th International Symposium on Insect-Plant Relationships, Neauchatel, Switzerland | Aug 17-21, 2014 |
| ▶ **Presentations** | |
| PhD excursion to Switzerland (Talk) | Oct 30, 2013 |
| 5th International Symposium on Insect-Plant Relationships, Neauchatel (Poster) | Aug 17-21, 2014 |
| 9th workshop on plant-insect interactions, Utrecht (Talk) | Nov 03, 2014 |
| Entomologendag, Ede, 2014 (Talk) | Dec 19, 2014 |
| ▶ **IAB interview** | |
| Meeting with a member of the International Advisory Board | Jan 05, 2015 |
| ▶ **Excursions** | |
| PhD scientific excursion to Switzerland | Oct 28- Nov 01, 2013 |
| *Subtotal Scientific Exposure* | *13.3 credits** |

| **3) In-Depth Studies** | *date* |
|---|---|
| ▶ **EPS courses or other PhD courses** | |
| Basic statistics | Dec 13-15 & 20-21, 2011 |
| Linear models | Jun 05,06 & 08, 2012 |
| ▶ **Journal club** | |
| PhD lunch meetings in Entomology | 2012-2015 |
| IPI meetings in Entomology | 2012-2015 |
| ▶ **Individual research training** | |
| *Subtotal In-Depth Studies* | *5.4 credits** |

| **4) Personal development** | *date* |
|---|---|
| ▶ **Skill training courses** | |
| Scientific Writing | Oct-Dec 2012 |
| PhD competence assessment | Mar 20 & Apr 19, 2012 |
| Techniques for writing and presenting a scientific paper | Apr 24-27, 2012 |
| Career Pespectives | Nov 13-Dec 11, 2014 |
| ▶ **Organisation of PhD students day, course or conference** | |
| ▶ **Membership of Board, Committee or PhD council** | |
| *Subtotal Personal Development* | *4.9 credits** |

| | |
|---|---|
| **TOTAL NUMBER OF CREDIT POINTS*** | **37.1** |

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

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

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