

Tritrophic interactions in wild and cultivated brassicaceous plant species

Rieta Gols

Promotoren:

Prof. dr. M. Dicke

Hoogleraar Entomologie, Wageningen Universiteit

Promotiecommissie:

Prof. dr. ir. R. Rabbinge

Dr. J.F. Stuefer

Dr. M. Macel

Dr. M. Heil

Wageningen Universiteit

Radboud Universiteit Nijmegen

Nederlands Instituut voor Ecologie, Heteren

Cinvestav, Campus Guanajuato, Mexico

Dit onderzoek is uitgevoerd binnen de CT de Wit Onderzoeksschool: Production Ecology and Resource Conservation (PE&RC)

Rieta Gols

Tritrophic interactions in wild and cultivated brassicaceous plant species

Proefschrift

ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit
Prof. dr. M. J. Kropff
in het openbaar te verdedigen
op woensdag 21 mei 2008
des namiddags te vier uur in de Aula

Gols, R. (2008)

Tritrophic interactions in wild and cultivated brassicaceous plant species

PhD-thesis Wageningen University - with references - with summary in Dutch

ISBN 978-90-8504-921-0

ABSTRACT

Plants have evolved a range of defence traits that prevent or reduce attack by insect herbivores. Direct defence traits hamper or reduce the performance and behaviour of the herbivores, whereas indirect defence promote the efficiency of natural enemies to attack the herbivores. Here, I focused on chemical plant defences, both direct and indirect, in brassicaceous plant species. Glucosinolates (GS), which are secondary plant compounds characteristic for the Brassicaceae, may not only affect growth and survival of herbivores, they may also reduce the performance of the herbivore's natural enemies such as parasitoids. Brassicaceous plant species, like most plant species, emit volatile organic compounds in response to herbivore feeding damage. These herbivore-induced plant volatiles (HIPV's) can be used by parasitoids of these herbivores to locate a host-infested plant. The production of HIPV's is a form of chemical indirect defence and is also studied here. The Brassicaceae family contains important crop plants such as cabbages and oilseeds. However, through artificial selection, the chemistry in crop plants may have been modified compared with their wild conspecifics in which defence traits are the result of natural selection. I compared the performance of herbivore and parasitoid species that differed in their food plant, respectively host specialisation, on several cultivated and wild conspecific plant populations. The specialist herbivore-parasitoid systems were not affected by the relatively high GS concentrations in leaf tissues of three mustard species (both wild and cultivated populations). However, the performance of even a specialist herbivore and its parasitoid differed on wild populations of *B. oleracea* compared to their performance on a *B. oleracea* cultivar. Development of a generalist herbivore and its parasitoid were even more strongly affected by differences in quality among the wild *B. oleracea* populations. Differences in plant quality affected not only pupal/adult mass and development time, as was found for the specialists, but also strongly reduced survival. Whether artificial selection has resulted in chemical changes with concomitant effects on the performance of their associated insects depends on the crop trait that has been selected for. Direct and indirect plant defences are usually investigated in separate studies. However, a conflict between direct and indirect defences may arise when a parasitoid female is attracted to a plant on which the development of her offspring is compromised. I studied the performance of two different parasitoid species on three different mustard species and also recorded their attraction to volatiles from host-infested plants. No conflicts between direct and indirect defences were revealed. Parasitoid females were attracted to plants on which their offspring was not negatively affected by the food plant quality of the host. Many studies comparing parasitoid attraction to HIPV's are conducted in Y-tube olfactometer or windtunnel experiments. However, under natural conditions parasitoids have to search for hosts in heterogeneous environments with different plant species that can be attacked by several herbivores. I observed parasitoid foraging behaviour in a slightly more complex set-up. Female *D. semiclausum* took more time to find a single host-infested *B. oleracea* plant in a set-up consisting of *B. oleracea* plants interspersed with a second brassicaceous plant species, *S. alba*, than in a monospecific set-up with only *B. oleracea* plants. Thus, *B. oleracea* plants may provide the herbivore with a chemical refuge against parasitism in the presence of highly attractive *S. alba* plants. Comparison of the foraging behaviour and population dynamics of hosts and their parasitoids in managed agricultural and unmanaged natural ecosystems may provide new insights into the mechanisms underlying tritrophic interactions and may help to reveal the importance of bottom-up and top-down control of arthropod herbivores.

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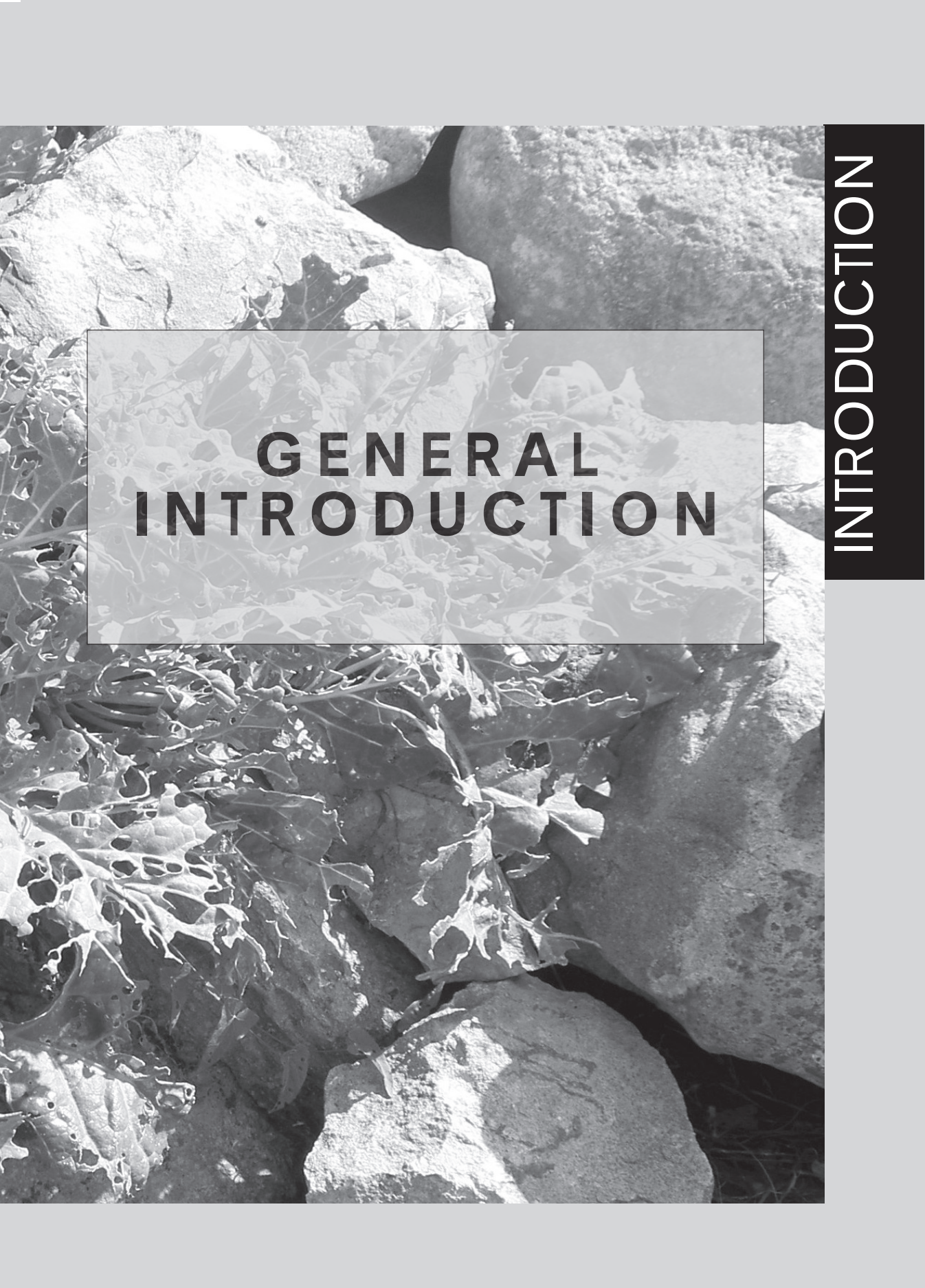
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A black and white photograph of a rocky coastline. In the foreground, there are large, light-colored rocks. To the left, there is a dense patch of seaweed with many small, circular holes, likely from sea urchins. A semi-transparent rectangular box is centered over the image, containing the text "GENERAL INTRODUCTION" in bold, black, sans-serif capital letters.

GENERAL INTRODUCTION

INTRODUCTION

1

Plant mediated effects in the Brassicaceae on the performance and behaviour of parasitoids

by R. Gols and J.A. Harvey

Direct and indirect plant defences are well studied, particularly in the Brassicaceae. Glucosinolates (GS) are secondary plant compounds characteristic in this plant family. They play an important role in defence against herbivores and pathogens. Insect herbivores that are specialists on brassicaceous plant species have evolved adaptations to excrete or detoxify GS. Other insect herbivores may even sequester GS and employ them as defence against their own antagonists, such as predators. Moreover, high levels of GS in the food plants of non-sequestering herbivores can negatively affect the growth and survival of their parasitoids. In addition to allelochemicals, plants produce volatile chemicals when damaged by herbivores. These herbivore induced plant volatiles (HIPV's) have been demonstrated to play an important role in foraging behaviour of insect parasitoids and brassicaceous plant species have been proven to be model systems in this regard. In addition, biosynthetic pathways involved in the production of HIPV's are being unraveled using the model plant *Arabidopsis thaliana*. However, the majority of studies investigating the attractiveness of HIPV to parasitoids are based on experiments mainly using crop plant species in which defence traits may have changed through artificial selection. Field studies with both cultivated and wild crucifers, the latter in which defence traits are intact, are necessary to reveal the relative importance of direct and indirect plant defence strategies on parasitoid and plant fitness. Future research should also consider the potential conflict between direct and indirect plant defences when studying the evolution of plant defences against insect herbivory.

Key words: crucifers, direct defence, glucosinolates, indirect defence, herbivore-induced plant volatiles

Introduction

Plants have evolved several strategies to reduce or prevent insect herbivory. These defence strategies can be divided into direct and indirect defences. Direct defences have a negative impact on development and behaviour of the herbivore, whereas indirect defences enhance the ability of natural enemies such as parasitoids and predators to exploit herbivores on the plant. Plant defences often involve the production of secondary chemical compounds (= allelochemicals) that can have negative effects on the development and survival of insect herbivores. Plant allelochemicals are often phylogenetically conserved in specific plant families or genera, such as glucosinolates (hereafter GS) in the Brassicaceae (Rosenthal and Berenbaum 1992; Schoonhoven et al. 2005). Chemicals that promote the effectiveness of natural enemies involve volatile compounds that are produced in response to herbivore feeding damage, so-called herbivore induced-plant volatiles (HIPV). These HIPV's are known to be attractive to parasitoids and predators of arthropod herbivores (Dicke 1999b; Turlings et al. 2002).

Parasitoids are insects that develop in or on the bodies of other arthropods, usually insects, whereas the adults are free living (Godfray 1994). Unlike predators that may need to feed on several prey to reach maturity, the development of parasitoids is dependent on the finite resources contained in a single host individual. Arthropod herbivores obtain their nutrition directly from their food plants. Consequently, parasitoids of these herbivores obtain their nutrition indirectly from the plants (Bottrell et al. 1998; Turlings and Benrey 1998). It has been shown that plant allelochemicals may not only negatively affect the development of herbivores but also that of their parasitoids (Hunter 2003; Ode 2006). Furthermore, parasitoid reproductive success is closely correlated with the female's ability to find hosts (Godfray 1994); therefore, parasitoids have evolved efficient foraging strategies to locate hosts in often complex environments. HIPV's have been demonstrated to play an important role in host-finding behaviour of parasitoids that attack larval stages of insect herbivores (Dicke 1999b; Turlings et al. 2002). Thus, plant-mediated effects on parasitoid performance and behaviour can act negatively through the production of allelochemicals and positively through the production of volatile attractants. However, as will be discussed, these two plant defence traits are not necessarily mutually exclusive (Havill and Raffa 2000; Hunter 2003).

The Brassicaceae family contains important crops such as cabbages (*Brassica oleracea*) and oilseeds (*B. nigra*, *B. napus*

etc.). A number of insect herbivores associated with these plants have become serious pests and as a result the interaction between these insects and their food plants is well studied. Parasitoid species are often restricted to a narrow range of host species, which has promoted the use of parasitoids to control insect pests in agricultural systems. Many studies have investigated the importance of HIPV's as foraging cues for parasitoids that attack hosts feeding on brassicaceous plant species. However, little is known about the effect of food-plant quality on parasitoid performance in this plant family. Plants in the Brassicaceae characteristically produce secondary plant compounds called GS. After tissue damage, myrosinases catalyse the hydrolysis of GS into (iso)thiocyanates and nitriles. A vast amount of literature has demonstrated that GS hydrolysis products are mobilised in defence against insect herbivores (Chew 1988; Rask et al. 2000). These compounds can act as feeding deterrents or significantly alter the physiology and development of some herbivores, through reduced growth rates, smaller adult size and increased mortality. Insect herbivores that mainly feed on brassicaceous plant species have evolved special adaptations to excrete and/or detoxify GS (Ratzka et al. 2002; Wittstock et al. 2004). Moreover, some insects sequester GS and employ them as defence against their own antagonists (Müller et al. 2001; Aliabadi et al. 2002;). The effect of brassicaceous plant species on the performance and behaviour of insect parasitoids is the topic of this review.

First, we examine the effect of brassicaceous food plant on the development of hosts and their parasitoids. We briefly describe the process of sequestration of GS by some insect herbivores. Because only a few studies have investigated the effect of GS-sequestration on organisms in the third trophic level, we examine this in both parasitoids and predators. Second, we review studies reporting on the attraction of parasitoids to HIPV. Furthermore, special attention is paid to the model plant *Arabidopsis thaliana* and its role in elucidating mechanisms underlying tritrophic interactions. Finally, we discuss the fact that most studies investigating tritrophic interactions in brassicaceous plant species use cultivated varieties. Artificial selection may have changed the chemistry of crop plants in such a way that the performance of herbivores and their parasitoids differ on cultivated and wild conspecific plants.

Direct plant defence: are herbivore and parasitoid performance differentially affected by plant quality?

GS are secondary plant compounds characteristic in the Brassicaceae and are considered to play an important role in the defence against herbivorous arthropods and pathogens (Chew 1988; Rask et al. 2000; Mithen 2001a). Plants containing GS have been shown to adversely affect the growth, development and survival of many insect herbivores. However, host plant chemistry can also have a negative impact on development of the natural enemies of the herbivores. Parasitoids of herbivores feeding on GS-containing plants may be negatively affected by plant-derived compounds that are stored into the haemolymph or other body tissues of the herbivore. Furthermore, the feeding strategy of the parasitoid larva may determine to what extent the parasitoid offspring are exposed to plant allelochemicals (Harvey and Strand 2002). For example, parasitoid species whose larvae are obligate tissue feeders and have to consume the host completely before pupation are likely to be exposed to plant-derived compounds that are stored in the host's body tissues or in the gut. Alternatively, parasitoids that selectively feed on haemolymph and fat body and pupate externally from the host larva may avoid exposure to harmful plant-derived compounds. Parasitoid performance may also be compromised due to reduced size or quality of the host itself. Furthermore, the efficiency of the host immune response may be reduced when the host is feeding on more toxic plant genotypes or species (Karimzadeh and Wright 2008), which in turn may increase parasitism success. In the following two sections, we will examine studies that have investigated the performance of parasitoids on hosts feeding on different species and populations of brassicaceous plants.

Interspecific plant variation

Table 1 provides an overview of studies that have investigated the performance of both herbivores and parasitoids reared on different brassicaceous plant species. Generalist herbivores, which can feed on plant species in several plant families, are usually more sensitive to plant allelochemicals than specialist herbivores, which are adapted to feed on plants containing specific phytotoxins, such as GS (Blau et al. 1978). Likewise, parasitoids attacking generalist herbivores have been shown to be more strongly affected by the herbivore's diet than parasitoids that attack hosts only feeding on brassicaceous plant species (Sznajder and Harvey 2003; Gols et al. 2008c). Specialist herbivores

have evolved efficient GS detoxification mechanisms (Ratzka et al. 2002; Wittstock et al. 2004). Consequently, parasitoids of specialist herbivores may be exposed to only low levels of GS or their breakdown products. However, the development of parasitoids of hosts that are specialised on brassicaceous plant species have also been shown to differ with host plant quality (Benrey et al. 1998; Harvey et al. 2003; Sznajder and Harvey 2003; Gols et al. 2007). Negative plant-mediated effects can even affect hyperparasitoids (parasitoids that develop in other parasitoids) in the fourth trophic level (Harvey et al. 2003; Soler et al. 2005). One factor to consider is that some brassicaceous plants contain allelochemicals, in addition to GS, such as cardenolides in the annual weed *Erysimum cheiranthoides* that are feeding deterrents to larvae of specialists such as *Pieris rapae* (Renwick 2001). Related species, like *Bunias orientalis*, are actually toxic to specialists in the Pieridae and this leads to precocious death of their parasitoids. However, the main GS in *B. orientalis* is sinalbin, which is not toxic to pierids, thus indicating the presence of some other, as of yet unidentified toxin (J.A. Harvey, unpublished).

Quite often, the performance of the host and its parasitoid are positively correlated (Benrey et al. 1998; Harvey et al. 2003; Sznajder and Harvey 2003), although the adverse effects of food plant characteristics on insect performance are usually less pronounced in the parasitoid than in the herbivore. Secretion and/or detoxification of plant allelochemicals by the host may dilute the effect of these compounds on the development of the parasitoid. In addition, the feeding strategy of the parasitoid larva may influence the extent to which parasitoid offspring are exposed to the adverse effects of plant-derived compounds. For example, Gols et al. (2008a) found that the haemolymph-feeding parasitoid *Cotesia glomerata* (Fig. 1b), developed equally well on hosts feeding on *Sinapis arvensis* and *Brassica nigra*, whereas its host, *Pieris brassicae*, was significantly smaller and took longer to complete development on *S. arvensis*. By contrast, Karowe and Schoonhoven (1992) showed that *C. glomerata* attained higher biomass and developed faster when parasitising *P. brassicae* caterpillars that feed on *Tropaeolum majus* than on *B. napus* and *B. oleracea*, whereas its host *P. brassicae* performed better on the latter two food plants. *Tropaeolum majus* contains GS, although it is not a member of the Brassicaceae. Differences in nutritional requirements and/or sensitivity to secondary plant metabolites may explain the differential performance of the host and its parasitoid.

Table 1: Plant-mediated effects on herbivore and parasitoid performance: interspecific plant variation in the Brassicaceae.

Plant species 1	Plant species 2	Plant species 3	Plant species 4	Herbivore species	Parasitoid species	Plant ranking: host high→low	Plant ranking: Parasitoid high→low	Reference
<i>Brassica oleracea</i>	<i>B. nigra</i>	<i>Barbarea vulgaris</i>		<i>Pieris brassicae</i>	<i>Cotesia glomerata</i>	1>2>3 DT* 2>1=3 PM*	1>2>3 DT 1≥2=3 AM*	Sznajder and Harvey 2003
<i>B. oleracea</i>	<i>B. nigra</i>			<i>P. brassicae</i>	<i>C. glomerata</i>		1=2 BS*, CM*	Harvey et al. 2003
<i>B. napus</i> Swedish turnip	<i>B. oleracea</i> sprouts	<i>B. napus</i> rape	<i>Tropaeolum majus</i>	<i>P. brassicae</i>	<i>C. glomerata</i>	1=2=3=4 S* 1>2=3=4 DT 1=2>3=4 AM	4≥2=1≥3 S 4>1=2=3 DT 4>2=1=3 L*	Karowe and Schoonhoven 1992
<i>B. oleracea</i> cv*	<i>Lunania annua</i>			<i>P. rapae</i>	<i>C. glomerata</i>	1>2 DT, PM	1>2 DT, HT*, S	Benrey et al. 1997
<i>B. oleracea</i> cv	<i>T. majus</i>	<i>L. annua</i>	<i>Cleome spinosa</i>	<i>P. rapae</i>	<i>C. glomerata</i>	1=2>3=4 DT	1>3=4=2 PAR* (in the field)	Benrey and Denno 1997
<i>B. oleracea</i>	<i>Sisymbrium officinale</i>	<i>Raphanus sativus</i>		<i>P. rapae</i>	<i>C. rubecula</i>	1=2>3 DT, AM	1=2>3 AM 1>2>3 DT	Harvey and Wagenaar 2006
<i>Sinapis alba</i> cv	<i>B. oleracea</i> cv			<i>Plutella xylostella</i>	<i>Diadegma semiclausum</i>	1>2 AM♀, 1=2 DT, AM♂	1>2 AM♂, DT♂; 1=2 AM♀, DT♀;	Gols et al. 2007
<i>B. rapa</i>	<i>Arabidopsis thaliana</i>			<i>P. xylostella</i>	<i>C. plutellae</i>	1=2 DT, PM, OV*, S	1=2 DT, SR* 1>2 HT, S	Barker et al. 2007
<i>B. pekinensis</i> cv	<i>B. oleracea</i> cv			<i>P. xylostella</i>	<i>C. plutellae</i>	1>2 DT; 1=2 S	2>1 PAR	Karimzadeh and Wright 2008
<i>B. oleracea</i>	<i>Barbarea vulgaris</i>	<i>B. nigra</i>		<i>Spodoptera exigua</i>	<i>C. marginiventris</i>	1>2>3 DT 1=2>3 PM	1≥2>3 DT 1>2=3 AM	Sznajder and Harvey 2003
<i>B. oleracea</i> cv collards	<i>B. nigra</i>	<i>B. oleracea</i> cv green cabbage		<i>Trichoplusia ni</i>	<i>Patrocloides montanus</i>		1=2=3 PAR; 1=2=3 SRF1*; 1>2=3; 1≥2≥3 SRF2*	Fox et al. 1996

*Explanation of abbreviations: cv=cultivar; AM= adult mass; BS= brood size; CM= cocoon mass; DT = □ paral

Intraspecific plant variation

Several studies have shown that GS are variably expressed in different cultivars (Rosa 1999; Kushad et al. 2004) and wild populations of *B. oleracea* (Mithen et al. 1995; Moyes et al. 2000; Gols et al. 2008c). Levels of GS are generally lower in *B. oleracea* cultivars than in wild populations (Mithen et al. 1995; Rosa 1999; Moyes et al. 2000; Kushad et al. 2004; Gols et al. 2008c). *Brassica oleracea* is an interesting plant species with respect to foliar GS composition in leaf tissues. The leaf tissues of this plant species sometimes contain up to ten different GS compounds, of which individual concentrations can differ considerably among different cultivars and wild populations. This is in contrast to other plant species in which one GS dominates the GS profile (e.g., sinigrin in *B. nigra* and sinalbin in *S. alba*). Wild populations of *B. oleracea*, which grow naturally along the Atlantic coastlines of England and France, differ considerably in constitutive and inducible levels of GS (Mithen et al. 1995; Moyes et al. 2000; Gols et al. 2008c). Aliphatic GS (GS derived from methionine) concentrations differed among the wild populations, but did not change in response to herbivore feeding (Gols et al. 2008c). Concentrations of indole GS (GS derived from tryptophan) have been demonstrated to be low in undamaged plants, but are differentially induced in response to feeding damage (Gols et al. 2008c).

The performance of herbivores feeding on plants differing in foliar GS content has been well studied (Hopkins et al. 1998; Li et al. 2000; Agrawal and Kurashige 2003). Plants with high levels of GS can reduce the performance of both generalist and specialist herbivores, although the effects of GS on specialists are usually less pronounced (Li et al. 2000; Gols et al. 2008b). Only a few papers have reported the effects of intraspecific variation in GS content on parasitoid performance (Table 2). For example, *P. rapae* developed faster and grew larger on one of three wild *B. oleracea* populations with the lowest level of inducible GS (Gols et al. 2008c). Development of both the solitary larval endoparasitoid, *C. rubecula*, and the gregarious pupal parasitoid, *Pteromalus puparum*, closely reflected plant quality for *P. rapae* feeding on the wild *B. oleracea* populations (Harvey et al. 2007a; Gols et al. 2008c). Interestingly, the generalist herbivore *Mamestra brassicae*, and its fairly specialised larval endoparasitoid, *Microplitis mediator* (Fig. 1a), developed more poorly on wild cabbage populations with the highest levels of constitutive GS (Gols et al. 2008c). Performance of the specialist herbivore, *P. rapae*, appeared to be affected by inducible indole GS in its food plant, whereas the generalist, *M. brassicae*, responded negatively to high overall GS concentrations. These results reveal variation in the differential

Table 2: Plant-mediated effects on herbivore and parasitoid performance: within plant variation in the Brassicaceae.

Plant species	Treatment 1	Treatment 2	Treatment 3	Parasitoid host species	Parasitoid species	Host ranking on treatments high→low	Parasitoid ranking on treatments high→low	Reference
<i>Brassica oleracea</i> populations	Old Harry	Winspit	Kimmeridge	<i>Pieris rapae</i>	<i>Cotesia rubecula</i>	1≥2≥3 PM*; 1>2=3 DT*	1=2≥3 AM*; 1=2=3 DT	Gols et al. 2008c
<i>B. oleracea</i> populations	Old Harry	Winspit	Kimmeridge	<i>P. rapae</i>	<i>Pteromalus puparum</i>	1≥2≥3 PM; 1>2=3 DT; 1=2=3 S*	1=2=3 BS*; DT, AM♀; 1>2=3 AM♂	Harvey et al. 2007a
<i>B. oleracea</i> populations	Cultivar	Kimmeridge	Old Harry	<i>Mamestra brassicae</i>	<i>Microplitis mediator</i>	1=2>3 S; 1>2>3 PM; 1=2>3 DT	1>2>3 S	Gols et al. 2008c
<i>Arabidopsis thaliana</i> ecotypes	Columbia	Lansberg erecta		<i>Plutella xylostella</i>	<i>C. plutellae</i>		1=2 DT, PM; S, OV*	Barker et al. 2007
<i>B. oleracea</i> cv nutrients	High N	Low N		<i>P. xylostella</i>	<i>Diadegma insulare</i>		1>2 SR*; 1≥2 AM	Fox et al. 1990, 1996
<i>B. oleracea</i> cv nutrients	High N	Low N		<i>Trichoplusia ni</i>	<i>Patrocloides montanus</i>		2<1 PAR* 1=2 SR	Fox et al. 1996
<i>B. nigra</i> root herbivory	No root herbivory	<i>Delia radicum</i> low density	<i>D. radicum</i> high density	<i>P. brassicae</i>	<i>C. glomerata</i>	1=3>2 DT 1=2=3 PW	1=3>2 DT 1>2=3 CM*	Soler et al. 2005
<i>B. nigra</i> shoot herbivory	No shoot herbivory	<i>P. brassicae</i>		<i>D. radicum</i>	<i>Trybliographa rapae</i>	1>2 S, AS	1>2 S, HT*	Soler et al. 2007a

Explanation of abbreviations: AM= adult mass; BS=brood size; CM= cocoon mass; DT = development time larva-pupa, larva cocoon; HT= Hind tibia length (indicator of size); PAR= rates of parasitism; S=survival to pupation/adulthood; SR=sex ratio, female bias.

effects of plant quality, possibly mediated through GS, on the development of oligophagous and polyphagous herbivores and their parasitoids.

Above, we referred to studies where food plant quality was compared in different strains or populations of the same species. However, the quality of an individual plant may change in response to a suite of biotic factors such as pathogen infections and herbivore feeding (Agrawal 1999a), as well as abiotic factors such as nutrient levels and light conditions (Schoonhoven et al. 2005). Over the course of a growing season, the quality of leaf tissues may change due in part to changes in abiotic conditions, leading to concomitant effects on herbivore-natural enemy interactions. This is particularly important for insects that have more than a single generation over the course of a year. One of the major shortcomings of current knowledge about the effects of GS on insect development in a multitrophic framework is that most of the studies have been based on experiments performed during a single time frame. Although many of the better-studied crucifers, including *A. thaliana*, *S. alba* and *B. nigra* are annuals with comparatively short life-cycles, other species, such as *B. oleracea* and *Bunias orientalis* are perennials that may live for 10 years or even longer (Blamey and Grey-Wilson 1989). Moreover, different populations of some annual species may grow at different times of the year. In this case, later generations of herbivores may develop on plants that differ profoundly in terms of their defence chemistry from the natal plants of their parents. For instance, the development of three cohorts of *P. xylostella* and its endoparasitoid *D. semiclausum* (Fig. 1c) differed significantly when reared on cultivated *B. oleracea* and *S. alba* plants grown over the course of a single summer and autumn season (Gols et

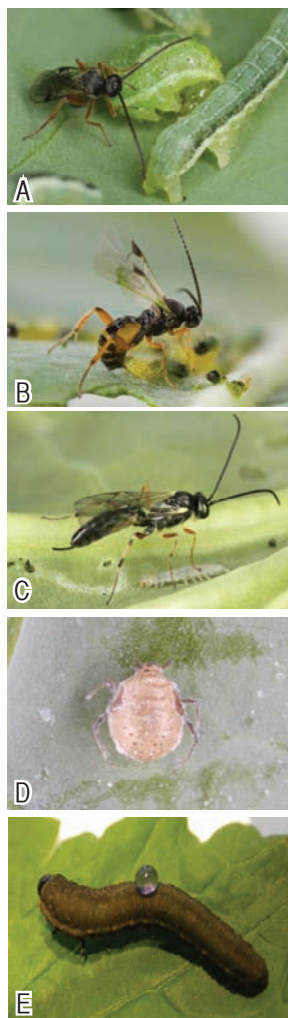


Figure 1: Several species of insect herbivores and parasitoids used in research on tritrophic interactions involving brassicaceous plant species. (A) *Microplitis mediator* is a fairly specialised solitary endoparasitoid of hosts that can feed on plants in several plant families. Here, a female is ovipositing in a larva of the cabbage moth, *Mamestra brassicae*. (B) *Cotesia glomerata* is also a fairly specialised gregarious larval endoparasitoid that attacks young larvae of pierid hosts. Here a female is parasitising a *Pieris brassicae* larva. (C) *Diadegma semiclausum* is a specialist solitary larval endoparasitoid of the diamondback moth, *Plutella xylostella*. This parasitoid can attack all four larval stages of its host. (D) A mummified aphid, *Brevicoryne brassicae*, containing a pupa of its parasitoid, *Diaeretiella rapae*. This parasitoid is a generalist and is known to attack a number of aphids including *Myzus persicae* and *Br. brassicae*. (E) A "bleeding" larva of the sawfly, *Athalia rosae*. Larvae of *A. rosae* sequester GS from the food plant in their haemolymph and easily bleed when touched by self-rupturing the first integument. This bleeding has been shown to deter predators of this herbivore (Foto credits: Fig. A-D bugsinthepicture.com, Fig. E Caroline Muller).

al. 2007). Although the plants were grown under strictly similar conditions in a greenhouse, foliar levels of GS changed quite dramatically from one experiment to another, with significant effects on the development of the herbivore and its parasitoid. This suggests that conditions in nature, which are not controlled, but which may change profoundly over quite short time scales, may affect associated consumers even more. Certainly, this is an understudied area that merits further investigation.

Thus far, we have described the results of studies investigating herbivore-parasitoid interactions in the above-ground compartment of a plant system. However, single plant, herbivore and parasitoid associations constitute only a small part of a complex above- and below-ground environment. Feeding damage caused by root and shoot herbivores may lead to both qualitative and quantitative changes in root and shoot tissues that are not necessarily correlated, due to differing effects of herbivory on primary and secondary metabolites in the below- and above-ground domains (Masters and Brown 1997; Bezemer and van Dam 2005). These changes in above- and belowground plant organs may not only affect the development of herbivores in the two spatially separated compartments, but also higher trophic level organisms associated with these herbivores. Low levels of herbivory by larvae of the cabbage root fly *Delia radicum* negatively influenced development of both the foliar herbivore *P. brassicae* and its parasitoid *C. glomerata* when reared on *B. nigra* plants (Soler et al. 2005). The adverse affects of root herbivory were even transmitted to the fourth trophic level; the hyperparasitoid *Lysibia nana* was significantly smaller when emerging from *C. glomerata* cocoons originating from plants exposed to root herbivory by *D. radicum* (Soler et al. 2005). The authors argued that increased concentrations of the GS sinigrin in foliar tissues of plants exposed to root herbivory could be responsible for the reduced performance of the above-ground insects. In a reciprocal study, Soler et al. (2007) investigated the effect of shoot herbivory on performance of *D. radicum* and its larval endoparasitoid *Trybliographa rapae*. Size and survival in both *D. radicum* and *T. rapae* were negatively affected when reared on plants whose shoots had been previously damaged by *P. brassicae* larvae, compared with undamaged control plants (Soler et al. 2007a). Shoot herbivory did not, however, affect nitrogen concentrations in the roots (Soler et al. 2007a). However, levels of the indole GS, glucobrassicin and neoglucobrassicin increased significantly in the secondary roots of plants in response to *P. brassicae* feeding. These studies show that by focusing exclusively on the above-ground environment, our understanding of the processes that

influence multitrophic interactions, e.g., between plants, herbivore and parasitoids, is likely to be incomplete (Harvey 2005).

The effect of sequestration of GS by insect herbivores on predators and parasitoids

Specialised insects feeding on brassicaceous plant species have not only evolved adaptations to cope with the adverse effects of GS, but some insects actively sequester GS in their haemolymph or other body tissues and use them for their own defence. Müller et al. (2001) reported that the sawfly *Athalia rosae*, which mainly feeds on GS-containing plants, concentrates GS obtained from the food plant in its haemolymph. The integument of *A. rosae* larvae is easily disrupted when touched and haemolymph is released from the wound. This 'easy bleeding' (Fig. 1e) has been shown to deter predators such as lizards (Vlieger et al. 2004), ants (Müller et al. 2002) and predatory wasps like *Vespula vulgaris* (Müller and Brakefield 2003). Ants were differentially deterred by pure GS compounds dissolved in a sucrose solution, but were more strongly deterred by diluted haemolymph in sucrose solution (Müller et al. 2002). Similarly, the foraging behaviour of *V. vulgaris* was more negatively affected by *P. rapae* caterpillars whose cuticle was treated with haemolymph from *A. rosae* than caterpillars whose cuticle was treated with pure sinalbin, the major GS in *S. alba* (Müller and Brakefield 2003). These results suggest that the repellent effect cannot be attributed to sinalbin alone and that different GS may differ in their deterrent activity against predators and other natural enemies.

The harlequin bug *Murganthia histrionica* also sequesters GS from its food plant and is distasteful to several species of bird predators (Aliabadi et al. 2002). The possibility of sequestration of GS by immature stages of *Pieris* species has generated controversy. An early study (Aplin et al. 1975) reported on the sequestration of GS by larvae and pupae of *P. rapae* and *P. brassicae*. However, a recent study by Müller et al. (2003), in which more advanced GS analysis techniques were used, found that GS could not be detected in body tissues or haemolymph of *P. rapae* and *P. brassicae* larvae that had been feeding on GS-containing food plants. Interestingly, Wiklund and Järvi (1982), demonstrated that starlings (*Sturnus vulgaris*) rejected *P. brassicae* caterpillars as food. Moreover, Rayor et al. (2007) reported that paper wasps (*Polistes dominulus*) spent more time handling *P. napi* caterpillars that had been feeding on *Erysimum cheiranthoides*, *Tropaeolum majus* and *B. nigra*, crucifers with

high levels of potentially deterrent GS, than *P. napi* caterpillars that had been feeding on a less toxic cabbage cultivar (*B. oleracea capitata*). Extended handling by the wasp involved removal of the gut, suggesting that, although GS might not be sequestered, the presence of plant tissue material in the gut reduces foraging efficiency.

GS become toxic through a process of catalytic hydrolysis by myrosinase enzymes, which are stored in cytoplasm of specialised myrosin cells that are scattered throughout the plant tissues (Mithen 2001a). Degradation of GS only occurs after cell rupture leading to the production of nitriles, isothiocyanates, thiocyanates and oxazolidinethiones. This myrosinase-GS defence mechanism is mirrored in the aphids *Brevicoryne brassicae* (abbreviated into *Br. brassicae*) and *Lipaphis erysimi* (Bridges et al. 2002). Both aphid species sequester GS from phloem sap in their host plant, but the enzyme myrosinase is endogenous to the aphids (Bridges et al. 2002; Kazana et al. 2007). When predatory larvae of the ladybird beetle *Adalia bipunctata* and the hoverfly *Episyrphus balteatus* fed on either *Br. brassicae* or *Myzus persicae*, survival was significantly lower on *Br. brassicae* (Francis et al. 2001b; Vanhaelen et al. 2002). *Myzus persicae* does not sequester GS, nor does it possess endogenous myrosinase. Moreover, for both predator species, survival depended on the host plant species on which *Br. brassicae* had been feeding previously. Survival of the predators was equally low on *S. alba* and *B. nigra*, but was higher when *Br. brassicae* had been feeding on *B. napus*, which contains much lower levels of total GS than the other two plant species. By contrast, survival of *A. bipunctata* was very high when the prey was *M. persicae*, irrespective of the food plant species on which the aphid had been feeding (Francis et al. 2001b). Chemical identification of GS and related degradation products in both aphid species revealed very low levels of GS and no degradation products in *M. persicae*, and very high levels of GS and isothiocyanates in *Br. brassicae* (Francis et al. 2001b). In a separate study Francis et al. (2001a) also found that other fitness correlates such as development time and adult body mass were not affected by food plant species of *M. persicae*. However, fecundity of *A. bipunctata* females that had been reared from *M. persicae* on *S. alba* was significantly lower than that of the same aphid species reared on *B. napus* with low GS levels. In contrast, this defence mechanism is not effective against, *Diaeretiella rapae* (Fig. 1d), a parasitoid of aphids feeding on several *Brassica* species. This parasitoid has a greater innate preference for *Br. brassicae* than for *M. persicae*, but differences in performance when reared on the two aphid

species were found to be minimal (Blande et al. 2004).

These results demonstrate the effectiveness of sequestration of food-derived GS by specialised herbivores against generalist natural enemies. In addition, the effectiveness of anti-predator activity often depends on the host plant on which the herbivore has been feeding. Plant-mediated effects against natural enemies are also present in less well adapted herbivores, but their effects can be compromised by the reduced performance of the herbivore itself.

Indirect defence: the production of volatile chemicals that attract parasitoids of herbivores feeding on brassicaceous plant species

When plants are damaged by herbivores, they emit volatile compounds (HIPV) that attract enemies of the herbivores such as parasitoids and predators (Dicke 1999b; Sabelis et al. 1999; Turlings et al. 2002). Many attempts have been made to elucidate which specific volatile plant compounds play a role in the attraction of parasitoids to their host plant. In addition, biosynthetic pathways involved in the production of the volatiles are being unraveled. *Brassica* cultivars, especially those of *B. oleracea*, are well studied with respect to HIPV and their attractiveness to the herbivore's natural enemies (see Table 3). All parasitoid species in Table 3 have been shown to discriminate between volatiles from damaged and undamaged plants and most of them are attracted more to HIPV than to volatiles emitted by artificially damaged plants. However, only a few parasitoid species prefer volatiles from host-damaged plants over plants damaged by non-host species (Geervliet et al. 1996; Shiojiri et al. 2000), although some parasitoid species can learn to differentiate between volatiles induced by host and non-host herbivores (Geervliet et al. 1998). These results indicate that volatile emissions are very similar when plants are damaged by different herbivores as has been demonstrated in earlier studies (Blaakmeer et al. 1994; Geervliet et al. 1997). Consequently, the parasitoids may be unable to detect these minor differences.

Among the volatile chemicals involved in host-plant location by parasitoids, green leaf volatiles and terpenoids are assumed to play an important role, because their levels change in response to herbivore feeding. However, these two groups of chemicals are ubiquitously produced in the plant kingdom and thus may not always provide reliable cues that reveal the

identity of the host-plant complex. Alternatively, the identity of the plant and its attacker can be determined based on differences in the relative concentrations of compounds in the volatile blends (de Boer et al. 2004). Physiological constraints usually limit the number of different host species that can be parasitised by a larval endoparasitoid, but herbivorous hosts specialised on brassicaceous plants can feed on a range of different plant species. Surprisingly, few studies have compared the composition of HIPV's and their attractiveness to parasitoids in different brassicaceous plant species (Takabayashi et al. 1998; Bukovinszky et al. 2005).

Many species in the Brassicaceae are short-lived annuals and often grow in disturbed areas associated with human activity (Feeny 1977). Therefore, herbivores and associated parasitoids have to find these plants growing in patches that are often spatially and temporally unpredictable. In addition, different generations of these insects may grow on different host plant species. Therefore, it is critically important for parasitoid females to be able to recognise a range of host food plants that may differ considerably in the composition of their volatile emissions when damaged by hosts. Volatile GS hydrolysis products seem obvious candidates as reliable signals in brassicaceous plant species. Attraction to GS hydrolysis products has thus far only been demonstrated in the aphid parasitoid *D. rapae* (Blande et al. 2007; Bradburne and Mithen 2000; Read et al. 1970). Volatile GS hydrolysis products in the headspace in cultivars of *B. oleracea* have been demonstrated to be very low (Blaakmeer et al. 1994; Geervliet et al. 1997; Bukovinszky et al. 2005), but they are higher and thus more detectable in the emissions of caterpillar frass (Agelopoulos et al. 1995). It would be interesting to analyse and quantify HIPV emissions and their attractiveness to parasitoids in wild *B. oleracea* populations in which foliar GS have been reported to be much higher than in cultivated strains.

Plant-herbivore-parasitoid interactions in domesticated and wild Brassicaceae

In the Brassicaceae, domestication has given rise to several important crops such as cabbages (*B. oleracea*, *B. napus*), oilseeds (*B. napus*, *B. juncea* and *B. rapa*) and mustard condiments (*B. nigra*, *S. alba*). Cultivation has generally been aimed at enhancing a trait or a suite of traits in the plant, such as the production of specific plant organs or structures. Importantly, this may or may not be compatible with direct or indirect defences. For instance,

one of the most important traits in vegetable crops is their taste. Selection on this trait may be in direct conflict with direct defence depending on whether this trait is emphasised (e.g., mustard condiments) or not (e.g., cabbage). As a consequence of artificial selection, levels of primary and secondary plant compounds in crop plants may have changed in such a way that these plants have become more susceptible to insect attack. For example, GS in *B. oleracea* cultivars are often reduced compared with wild conspecific plants (Rosa 1999; Moyes et al. 2000; Kushad et al. 2004). Several studies have demonstrated that both herbivores and their parasitoids perform more poorly on wild than on cultivated brassicaceous plant species (Benrey et al. 1998; Harvey et al. 2007a; Gols et al. 2008c).

Resistance to and persistence of pesticides in the environment have promoted the search for alternative methods to control pests in agricultural systems. Many studies in agro-ecological research assume that tritrophic interactions (e.g., plant-herbivore-parasitoid interactions) function similarly in managed and unmanaged ecosystems (Barbosa 1993). However, the validity of these assumptions has rarely been empirically tested. In a seminal paper, Root (1973) introduced the 'resource concentration' hypothesis, which states that specialised herbivores attain higher densities in monocultures, because they are more likely to find and remain on host plants that are growing in pure stands. Likewise, given that they evolved in more complex natural ecosystems, parasitoids are often less challenged in finding hosts in agricultural monocultures and frequently overexploit host populations in cropping systems. Furthermore, the resource concentration hypothesis may also apply to generalist herbivores as a consequence of reduced levels of allelochemicals. This may explain why generalist herbivores, such as the cabbage moth (*Mamestra brassicae*) are not easy to find in natural plant assemblages but often become abundant, damaging pests in cabbage fields. Comparison of the foraging behaviour and population dynamics of hosts and their parasitoids in managed agricultural and unmanaged natural ecosystems may provide new insights into the mechanisms underlying tritrophic interactions (e.g., Ohsaki and Sato 1999).

For many crop plant species it has been demonstrated that herbivore-damaged plants emit volatile compounds that are attractive to parasitoids (for examples of parasitoid attraction in the Brassicaceae, see Table 3) and some parasitoids can even discriminate between volatiles emitted by different cultivars of the same crop species (Geervliet et al. 1996; Liu and Jiang 2003). However, plant breeding aimed at specific traits may

Table 3: Parasitoid attraction to brassicaceous plant- and host-related volatiles.

Plant	Treatment	Preference ranking most preferred → least preferred	Reference
<i>Cotesia glomerata</i> - <i>Pteris brassicae</i>			
<i>Brassica oleracea</i> cv*	<i>Pteris brassicae</i>	PHC*>DP*>AD*>F*>H*>C*	Steinberg et al. 1993
<i>B. oleracea</i> cv	Different hosts: <i>P. brassicae</i> , <i>P. rapae</i> , <i>P. napi</i> , <i>Mamestra brassicae</i> , <i>Plutella xylostella</i> , <i>Aporia crataegi</i>	Different PHC's versus C: PHC's > C Different PHC combinations: no preference	Geervliet et al. 1996
<i>B. oleracea</i> cv	Host regurgitant and artificial damage	PHC>C, ADR*>AD>R*=C	Mattiacci et al. 1994
<i>B. oleracea</i> cv	β-glucosidase (βgluc) and artificial damage	AD+βgluc= ADR >AD> βgluc=C	Mattiacci et al. 1995
<i>B. oleracea</i> cv	Duration of feeding		Scascighini et al. 2005
<i>B. nigra</i>	Root herbivory	C>RH*; PHC=PHC+RH	Soler et al. 2007b
<i>B. oleracea</i> cv	Damage by parasitised / unparasitised hosts; regurgitant and artificial damage,	ADR-unpar* (<i>P. brassicae</i>)>ADR-par* (<i>P. brassicae</i>); ADR-unpar(<i>P. rapae</i>)=ADR-par(<i>P. rapae</i>)	Fatouros et al. 2005
<i>Cotesia glomerata</i> - <i>Pteris rapae</i>			
<i>B. oleracea</i> cv	Host and non-host (<i>P. xylostella</i>) damage, artificial damage	DP-host=AD>C; DP-host=DP-non-host; PD(host and non-host)>DP-host	Shiojiri et al. 2000
<i>Florippa indica</i> , <i>Erigeron annuus</i> , <i>Digitaria ciliaris</i>	Host and artificial damage, different undamaged plant species	<i>R. indica</i> : DP, AD>C; <i>R. indica</i> > <i>E. annuus</i> , <i>D. ciliaris</i>	Takabayashi et al. 1998
<i>B. oleracea</i> cv, <i>Lunaria annua</i> , <i>Tropaeolum majus</i> , <i>Cleome spinosa</i> <i>Arabidopsis thaliana</i> <i>A. thaliana</i>	Different plant species, undamaged different transgenic lines with modified HPL activity Mutant with reduced (Z)-3-hexenal production, synthetic green leaf volatiles	<i>B. oleracea</i> > <i>L. annua</i> > <i>T. majus</i> > <i>C. spinosa</i>	Benrey et al. 1997, 1998
<i>Cotesia rubecula</i> - <i>Pteris rapae</i>			
<i>B. oleracea</i> cv	Host and artificial damage, hosts, host products	PHC>HD=AD>F>H>C	Shiojiri et al. 2006a
<i>B. oleracea</i> cv	Different hosts: <i>P. rapae</i> , <i>P. brassicae</i> , <i>P. napi</i> , <i>M. brassicae</i> , <i>P. xylostella</i>	Different PHC's versus C: PHC's > C: different PHC combinations: no preference; H(<i>P. rapae</i>)=H(<i>P. napi</i>) >H(<i>P. brassicae</i>), F(wet)>F(dry)	Agelopoulos and Keller 1994bc; Agelopoulos et al. 1995; Geervliet et al. 1994
<i>B. oleracea</i> cv	Damage by parasitised / unparasitised hosts; regurgitant and artificial damage	PHC-unpar>PHC-par; ADR-par>ADR-unpar for both <i>P. rapae</i> and <i>P. brassicae</i>	Fatouros et al. 2005
<i>A. thaliana</i>	Host damage, artificial damage	PHC>AD>C	van Poecke et al. 2001
<i>A. thaliana</i>	Different host: <i>P. rapae</i> , <i>P. xylostella</i> , <i>Spodoptera exigua</i> , <i>Tetranychus urticae</i> , <i>Myzus persicae</i>	PHC's versus C: PHC's>C except PHC (<i>M. pers</i>) =C; PHC's versus PHC(<i>P. rap</i>): PHC= PHC(<i>P. rap</i>) except: PHC(<i>P. rap</i>)> PHC (<i>M. pers</i>)	van Poecke et al. 2003

<i>A. thaliana</i>	jasmonic acid, salicylic acid, different transgenic lines (<i>NahG</i> and <i>Lox2</i>)		van Poecke and Dicke 2002
<i>C. plutellae</i> - <i>Plutella xylostella</i>			
<i>B. napus</i> cv	Host and artificial damage, host products	PHC=DP=AD>H=C	Potting et al. 1999
<i>B. campestris</i> cv, <i>B. oleracea</i> cv	Host and artificial damage,	<i>B. camp</i> : PHC=DP>AD>C; <i>B. oler</i> : PHC=DP=AD>C; PHC(<i>B. camp</i>)>PHC(<i>B. oler</i>)	Shiojiri and Takabayashi 2003; Shiojiri et al. 2000
<i>B. oleracea</i> cv, <i>Raphanus sativus</i>	Host and non-host (<i>P. rapae</i>) damage, artificial damage	both plant species: DP-host>DP-non host, AD, C; on cabbage: DP-host>DP(host and non-host)	Shiojiri et al. 2000
<i>B. oleracea</i> cv's	Host damage + elevated CO ₂ -levels, different cultivar; Lennox and Rinda	Lennox and Rinda: PHC's>C; Lennox: (PHC+CO ₂)>C; Rinda: (PHC+CO ₂)=C	Vuorinen et al. 2004
<i>B. oleracea</i> cv	Host damage + O ₃	PHC, (PHC+O ₃)>C; PHC>(PHC+O ₃)	Pinto et al. 2007
<i>Diadegma semiclausum</i> - <i>Plutella xylostella</i>			
<i>B. oleracea</i> cv	Host damage, artificial damage, host products	PHC, DP, AD>C; DP>AD, C>host products on dummy plant	Ohara et al. 2003ab
<i>B. oleracea</i> cv	Host damage, host, feces	PHC, F>B*; H=B	Roßbach et al. 2005
<i>B. oleracea</i> cv, <i>Sinapis alba</i> cv; <i>Hordeum vulgare</i> cv	Different plant species with and without host damage	C(<i>S. alba</i>)>C(<i>B. oler</i>)>C(<i>H. vulg</i>); PHC(<i>B. oler</i>), PHC(<i>S. alba</i>)>C; PHC(<i>B. oler</i>) = C(<i>S. alba</i>)	Bukovinszky et al. 2005
<i>Diadegma mollipla</i> - <i>Plutella xylostella</i>			
<i>B. oleracea</i> cv	Host damage, hosts, feces	PHC, F, H=B	Roßbach et al. 2005
<i>Diaeretiella rapae</i>: <i>Myzus persicae</i>/ <i>Brevicoryne brassicae</i>/ <i>Lipaphis erysimi</i>			
<i>A. thaliana</i>	Host damage: <i>M. persicae</i>	PHC=C (naïve wasps), PHC>C (experienced wasps)	Girling et al. 2006
<i>B. rapa</i> cv	Host damage: <i>M. persicae</i>	PHC=C (naïve and experienced wasps)	Blande et al. 2007
<i>B. oleracea</i> cv	Host: <i>M. persicae</i>	H=B	Read et al. 1970
<i>B. oleracea</i> cv	Host: <i>Br. brassicae</i>	H>B, C>B	Read et al. 1970
<i>B. rapa</i> cv	Host damage: <i>L. erysimi</i>	PHC=C (naïve wasps), PHC>C (experienced wasps)	Blande et al. 2007
<i>B. oleracea</i> near isogenic lines	Isothiocyanates, <i>B. oleracea</i> juice Glucosinolate composition	allyl ITC>B; <i>B. oler</i> -juice>B 2-Propenyl line, 3-butenyl line>B; 3-butenyl line>2-propenyl line	Read et al. 1970 Bradburne and Mithen 2000
	Isothiocyanates, pure compounds	3-butenyl ITC>B; 4-pentenyl ITC=B; 3-butenyl ITC=4-pentenyl ITC	Blande et al. 2007

*Explanation of abbreviations: AD= artificial damage; ADR= artificial damage plus host regurgitant; B=blank; C= undamaged control plants; cv= cultivar; DP= host-damaged plants with host and its products removed; F=feces; H=host; par=parasitised host; PHC= plants host complex; host-damaged plants with the host and its products present; R= host regurgitant; RH= plant damage by root-herbivores; unpar=unparasitised host.

not only have changed levels of nutrients and GS but also the quality and/or quantity of volatile blends produced in response to feeding damage. It would be interesting to compare the attraction of parasitoids to cultivated and conspecific wild plants and to determine the chemical composition of the volatiles blends emitted by these plants

Because they are adapted, specialist herbivores have evolved efficient GS detoxification systems and larvae of these species may use GS even as feeding stimulants. Consequently, the negative effects of GS on the performance of specialist herbivores are considered to be low. However, high GS concentrations may also affect negatively the development of specialist herbivores (Agrawal and Kurashige 2003). Therefore in natural plant populations where individual plants or populations contain high levels of GS, it is expected that the development of specialists may also be compromised. Finally, individual GS compounds may differ in their defence activity. Reduced levels of GS in cultivated Brassicaceae may lead to underestimations in the adverse effects of GS when compared with wild conspecifics, in which levels of toxic secondary plant compounds have not been modified.

***Arabidopsis* and herbivore-parasitoid interactions**

The model plant *A. thaliana* is also a member of the Brassicaceae. This plant species has been used for a wide range of research topics such as plant development, pathogen resistance and secondary plant metabolite chemistry. As a member of the Brassicaceae, *Arabidopsis* also synthesises GS. At present, the elucidation of pathways involved in the biosynthesis of GS, especially aliphatic GS, relies substantially on research with *Arabidopsis* (Halkier and Gershenzon 2006). Since GS hydrolysis products play a role in plant defence against insects, the effects of GS on herbivore performance have recently been studied using different genotypes/mutants of this plant species (Stotz et al. 2000; Lambrix et al. 2001; Kliebenstein et al. 2002; Burow et al. 2006). For example, Kliebenstein et al. (2002) and Burow et al. (2006) investigated the effect of an epithiospecifier protein (EPS) on the performance of generalist and specialist lepidopteran herbivores. This ESP generates the production of nitriles instead of the more toxic isothiocyanates. This raises the question as to why a plant would redirect its defence chemistry and become more susceptible to insect herbivory. Burow et al.

(2006) suggest that the expression of ESP genes may play a role against other attackers such as pathogens, or else plants expressing ESP may be more attractive to insect parasitoids. An alternative explanation for the existence of ESP could be that selection against the adverse effects of ESP expression is very low in *Arabidopsis* populations. *Arabidopsis* is a winter annual, which starts to flower in March and completes its development in April to early May. Most potential herbivores, such as *P. rapae*, usually first appear in April and May when *Arabidopsis* plants are fully seeded and when the plant's vegetative tissues have disappeared. A temporal mismatch between the phenology of *Arabidopsis* and insect herbivores such as *P. rapae* suggests that we should interpret the adaptive significance of defensive traits in this plant with some caution (Yano 1994; Arany et al. 2005; Harvey et al. 2007b). Defensive traits in *Arabidopsis* may be phylogenetically conserved in the genome, and there may be little cost in maintaining them.

The availability of many mutant and transgenic lines has extended the study of indirect defences (i.e., the production of plant volatiles that attract natural enemies of insect herbivores) to *Arabidopsis*. Similar to other brassicaceous plant species, *Arabidopsis* emits volatile chemicals in response to herbivore feeding that are attractive to parasitoids (van Poecke et al. 2001, 2003; Girling et al. 2006;). Genes from major biosynthetic pathways are induced in response to *P. rapae* feeding (van Poecke et al. 2001). These genes include *AtTPS10*, *AtPAL1*, *AtLOX2*, *AtHPL*, and *AtAOS*. *AtTPS10* is a terpenoid synthase involved in myrcene production and *AtPAL1* encodes phenylalanine ammonia-lyase, which plays a role in methyl salicylate production. *AtLOX2* and *AtHPL*, encoding lipoxygenase and hydroperoxide lyase, respectively, and both are important for the production of green leaf volatiles (C_6 alcohols, aldehydes and esters). *AtAOS* encodes allene oxide synthase. Both *AtAOS* and *AtLOX2* play a role in the production of jasmonic acid, a plant hormone that plays an important role in induced direct and indirect plant defence responses (Karban and Baldwin 1997). *Arabidopsis* mutants were used to elucidate signal transduction pathways and the role of the hormones jasmonic acid and salicylic acid in induced indirect plant defences (van Poecke and Dicke 2002). Mutants of this plant species that were impaired in the octadecanoid and salicylic pathway were less attractive to the parasitoid *C. glomerata* than wild-type plants.

Transgenic lines with enhanced hydroperoxide lyase (HPL) activity produced more (*Z*)-3-hexanal when damaged by *P. rapae* and were more attractive to *C. glomerata* than wild-type

Arabidopsis plants (Shiojiri et al. 2006b). An *Arabidopsis* mutant (*alB4*), in which the production of (*Z*)-3-hexanal was suppressed, attracted fewer *C. glomerata* females when damaged by *P. rapae* than wild-type plants (Shiojiri et al. 2006a). Moreover, *C. glomerata* females were also attracted to synthetic green leaf volatiles (Shiojiri et al. 2006a). Contrastingly, *C. plutellae* females did not discriminate between *alB4* and wild type plant when damaged by its host *P. xylostella* (Shiojiri et al. 2006a). These results suggest that the relative importance of green leaf volatiles as parasitoid attractants is species-specific.

Mutant and transgenic lines of *A. thaliana* can be used to investigate the relative role of volatile chemicals as natural enemy attractants. However, results should be verified using the plant species on which these herbivores and their parasitoids naturally occur. In addition, although many studies have reported on parasitoid attraction to herbivore-induced *A. thaliana* volatiles, only one has examined the quality of the host for the parasitoid when reared on *Arabidopsis* (Barker et al. 2007). In this study, adult *C. plutellae* were significantly smaller when the host, *P. xylostella*, was reared on *A. thaliana*, than when the host was reared on *B. rapa*.

Future directions

In this review we have reported studies showing that herbivores and their parasitoids are differentially affected by the herbivore's food plant and that wild brassicaceous plant species tend to be more toxic than cultivated strains or species. Very few studies have actually shown that GS hydrolysis products result in reduced development of the herbivore (Agrawal and Kurashige 2003). The compartmentalised myrosinase-GS defence in the Brassicaceae complicates empirical testing of the adverse effects of GS. Since the biosynthesis of aliphatic GS is well-studied, the effect of GS on insect herbivores and their parasitoids is mainly restricted to aliphatic GS (GS derived from methionine) and to a lesser extent the aromatic GS (GS derived from phenylalanine). Indole GS, which are derived from tryptophan, could also play a role in defence against insects (Soler et al. 2007a; Gols et al. 2008c). In addition, differences in the expression of myrosinase enzymes, which catalyze hydrolysis of GS, could differ among plants species and populations and affect herbivore and parasitoid performance.

Insect growth and development also depend on the availability of primary metabolites such as amino acids that are

often limiting. It is likely that levels of primary metabolites vary among plants species. Artificial diets (e.g., Agrawal and Kurashige 2003) or the use of mutant plants that have been modified to emphasise a single trait may help to better understand the relative effect of GS on insect performance, but one should also bear in mind the effect of other defence-related compounds as well as nutrients.

Little is known about the ability of parasitoids to detoxify allelochemicals (see also Ode 2006). The alimentary tract of endoparasitoid larvae is not externally connected until after egression and pupation, and thus any phytochemicals ingested during larval feeding must be stored until they are voided in by-products such as meconia and/or cocoon silk (Barbosa et al. 1986; Bowers 2003). Specialist herbivores feeding on brassicaceous plants are adapted to feed on plants containing GS and have evolved special detoxification mechanisms (Ratzka et al. 2002; Wittstock et al. 2004). Parasitoid larvae may have developed their own detoxification mechanism(s), or else they can tolerate high levels of allelochemicals and might even sequester them. Some studies have investigated the detoxification of GS in predators of insect herbivores. Feeding on *M. persicae* and *Br. Brassicae*, induced glutathione S-transferase activity in the hoverfly *Episyrphus balteatus* (Vanhaelen et al. 2001). Glutathione S-transferase is an important detoxification enzyme in eukaryotes. Other enzymes such as cytochrome P450's can also metabolise xenobiotics and could play a role in detoxification of plant-derived allelochemicals. The effect of GS and other allelochemicals on physiological traits of parasitoids is also virtually unknown. In particular, it would be interesting to determine what effects (if any) GS in the host's diet have on the ability of parasitoids to utilise resources for maintenance and reproduction (Jervis et al. 2008).

The majority of studies investigating the attractiveness of HIPV to parasitoids are based on windtunnel or Y-tube olfactometer experiments, mainly using crop plant species (Table 3) (for a critical review see Hunter 2002). However, little is known about the spatial scale at which these plant volatiles are perceived by parasitoids in the field. Moreover, in natural habitats parasitoids have to forage in complex environments that may consist of many different plant species where there are structural and chemical barriers. The foraging behaviour of parasitoids has been demonstrated to be affected by the structure and identity of the local plant community (Gols et al. 2005; Bukovinszky et al. 2007). In addition, individual foraging decisions do not necessarily translate directly into differences in population dynamics. For example, parasitism rates by *C. plutellae* differed when the

parasitoids were reared on two different host plants in short-term experiments, but in long-term experiments the population dynamics were similar on both host plant species (Karimzadeh et al. 2004). Field studies are thus necessary to reveal the relative importance of HIPV on plant/parasitoid fitness in both managed agricultural and unmanaged natural ecosystems (Geervliet et al. 2000).

One other area that is understudied is the degree of local adaptation exhibited by herbivore and natural enemy populations to different crucifer species that grow in their habitats. This is especially important in understanding how consumers respond to invasive plants, given that several species in the Brassicaceae are considered to be highly invasive pests in North America and Europe (Dietz et al. 1999; Meekins and McCarthy 1999; Lankau and Strauss 2007;). Recent empirical evidence suggests that plants with novel secondary chemistries have the capacity to invade new regions because the local herbivore populations are not adapted to them (Cappuccino and Carpenter 2005; Keeler et al. 2006; Callaway and Vivanco 2007). Furthermore, studies with invasive plants have rarely included natural enemies of herbivores associated with these plants. Studies with invasive crucifers offer an excellent opportunity for understanding mechanisms underlying novel interactions, and how other processes such as 'ecological fitting' (Agosta 2006) may determine the success of insects associated with invasive plants.

In this review we have focused on the effect of the host's food plant (so-called direct plant defences) and on the effects of plant volatile emissions (so-called indirect plant defences) on the development and behaviour of insect parasitoids and predators. In most cases, these two plant defence strategies have been studied independently. However direct and indirect defences may not act independently from each other and this may impose an evolutionary 'dilemma' or 'conflict' when a female parasitoid is attracted to a plant that negatively influences the development of her progeny (Dicke 1999c; Havill and Raffa 2000; Hunter 2003). Future research should consider this potentially important conflict when studying the evolution of plant defences against insect herbivory.

Acknowledgements

We would like to thank Caroline Müller for the invitation to write this review and Marcel Dicke for giving constructive comments on draft versions of this paper. Pictures were kindly provided by Tibor Bukovinszky, Hans Smid and Caroline Müller.

2

Thesis outline

by R. Gols

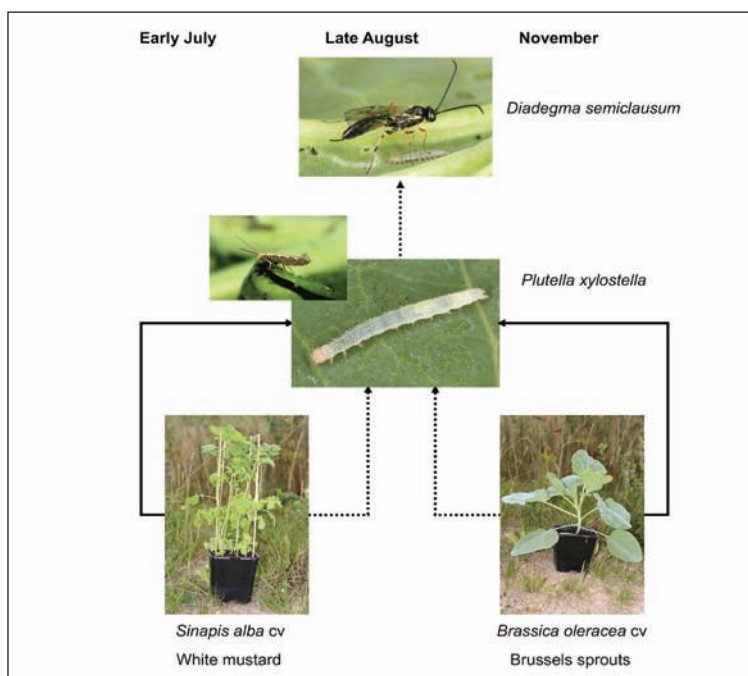
Plants can defend themselves in various ways. Two major types of defences are direct and indirect defence. Direct defences affect development and behaviour of the herbivores, whereas indirect defences refer to plant traits that promote herbivore exploitation by natural enemies such as parasitoids and predators. In my thesis I focus on chemical plant defences, i.e., the production of allelochemicals (direct defence) and volatile organic compounds that are produced in response to herbivore damage and are attractive to the herbivore natural enemies (indirect defence).

The Brassicaceae provide an excellent plant family to study chemical defences. All brassicaceous plant species biosynthesise glucosinolates (GS). These compounds have been demonstrated to play a role in defence against generalist herbivores (Chew 1988; Louda and Mole 1991; Mithen 2001), whereas specialist herbivores have evolved efficient detoxification or secretion mechanisms to circumvent toxic effects of GS (Ratzka et al. 2002; Wittstock and Gershenzon 2002). However, high levels of GS may also impact negatively on the performance of specialist herbivores (Li et al. 2000; Agrawal and Kurashige 2003). In addition, toxic secondary plant compounds such as GS may not only reduce the development of herbivores but also that of their parasitoids (Hunter 2003; Ode 2006). The effects of GS on parasitoids developing inside hosts feeding on brassicaceous plants have received little attention in the literature (Harvey et al. 2003) and this is one of my thesis objectives.

Artificial selection in the Brassicaceae has given rise to important crops such as cabbages (*Brassica oleracea*) oilseeds (e.g., *B. nigra*, *B. juncea*) and mustard condiments (e.g., *B. nigra*, *Sinapis alba*). Resistance to and persistence of pesticides in the environment have promoted the search for alternative methods to control pests in agricultural systems. The role of herbivore-induced plant volatiles (HIPV) in host-finding behaviour of parasitoids that attack larval stages of insect herbivores is well studied in brassicaceous crop plant species (Table 3, Chapter 1).

Thus, plant-mediated effects on parasitoid performance and behaviour can act negatively through the production of phytotoxins (direct defence) and positively through the production of volatile attractants (indirect defence). These two plant defence traits are not necessarily mutually exclusive (Havill and Raffa 2000; Hunter 2003). For instance, the plant hormone jasmonic acid plays an important role in the induction of both direct and indirect plant defences (Dicke and van Poecke 2002). In other words, a plant with high levels of direct defences may also have high levels of indirect defences and *vice versa*. A conflict may arise when a herbivore's enemy is attracted to plants that have a negative impact on its own development or that of its progeny (Havill and Raffa 2000). Yet, plants may also differentially control direct and indirect defences (Kahl et al. 2000). One of the most challenging issues in the study of plant-insect interactions is to determine how this conflict is resolved. However, it has been a longstanding practice to study direct and indirect plant defences independently. This potential conflict between direct and indirect plant defences is the subject in part II.

Figure 1: Temporal changes affect plant chemistry and tritrophic interactions. Host-plant quality was evaluated over the course of a growing season for *P. xylostella* and its larval endoparasitoid *D. semiclausum*. A cultivar of *B. oleracea* and *S. alba*, respectively, were chosen as food plants since they are known to differ qualitatively in glucosinolate content.



Plant breeding programs may have disrupted the original plant defence strategies that were present in the wild progenitors of cultivated species. Consequently, changes in plant chemistry, mediated by artificial selection, may influence the behaviour and development of consumers over several trophic levels. Most importantly, in wild plants defence mechanisms have not been constrained by the "directional selection" that characterises

many crop plants. Therefore, to understand the evolution of plant defences against insect herbivores, multi-trophic interactions should also be studied in wild conspecifics of the cultivated plant species, where plant defences are the likely result of a range of biotic and abiotic selection pressures.

In this thesis, I investigate direct and indirect plant defences in wild and cultivated brassicaceous plant species and their effects on associated lepidopteran herbivores and their larval endoparasitoids. The herbivores and the parasitoids differed in food plant and host specialisation, respectively. I monitored the

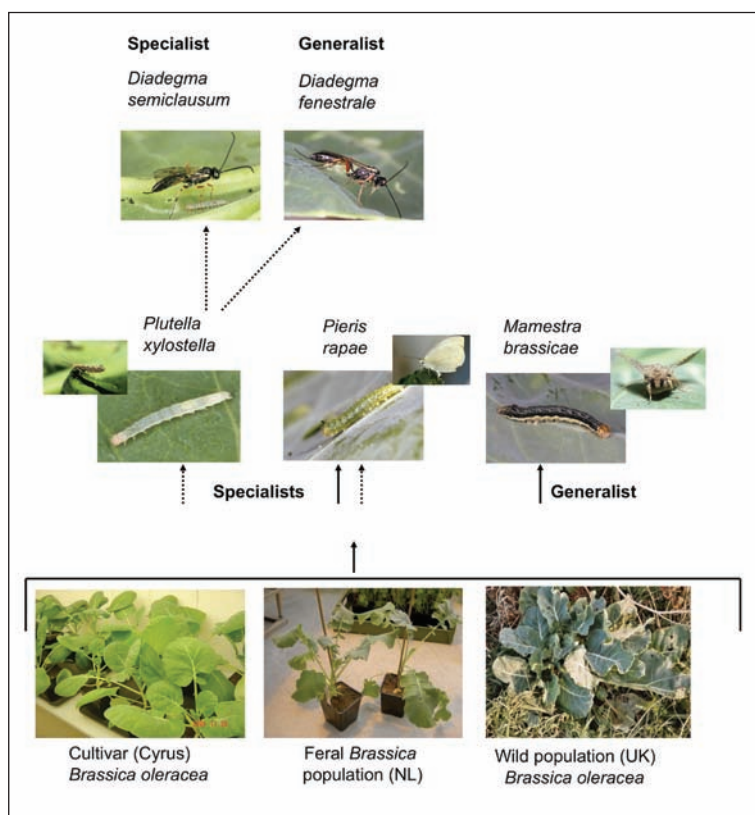
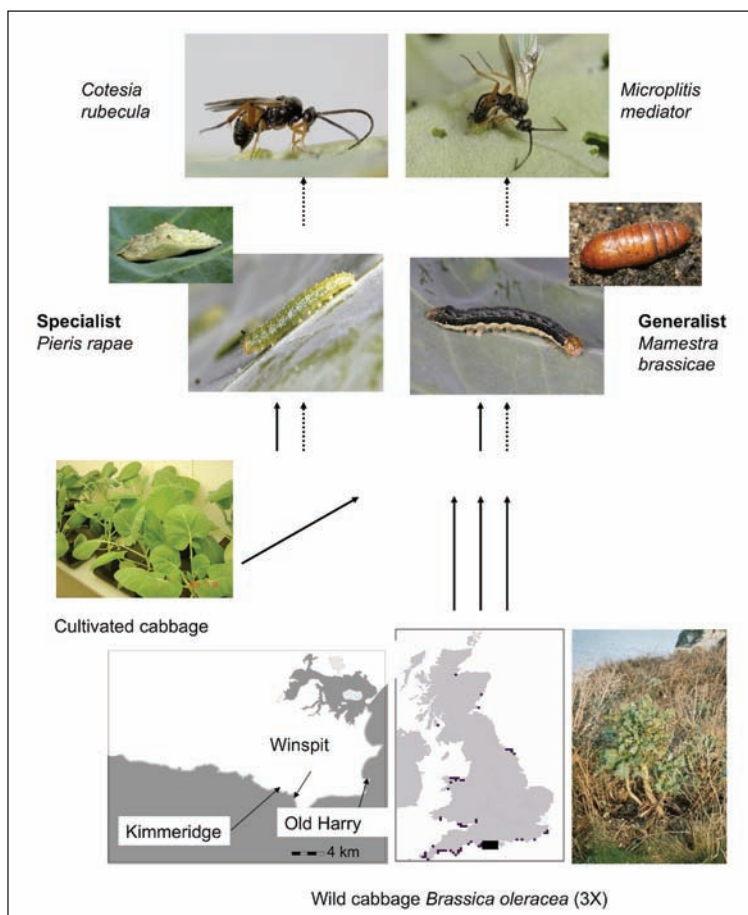


Figure 2: Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild *Brassica* populations. Through artificial selection, domesticated plants often contain reduced levels of phytochemicals compared to their wild progenitors. The development of several species of herbivores and endoparasitoids was investigated when reared on three *Brassica* populations that differ in their degree of domestication. Since generalists tend to be more sensitive to plant toxins than specialists, I included herbivores and parasitoids differing in their degree of food plant and host specialisation, respectively.

development of specialist herbivores (*Pieris brassicae*, *P. rapae*, and *Plutella xylostella*) and a generalist herbivore (*Mamestra brassicae*), which are all pest species on brassicaceous crop plants. The parasitoid species (*Cotesia glomerata*, *C. rubecula*, *Diadegma semiclausum*, *D. fenestrale* and *Microplitis mediator*) are all larval endoparasitoids, i.e., the parasitoid larvae develop in a still growing host.

The thesis is divided into three parts. In Part I, plant-mediated effects on herbivore and parasitoid performance are investigated. Plant quality does not only influence performance (survival,

Figure 3: Genetic variation in the defence chemistry of wild cabbage populations from the Atlantic coast of the UK and its effects on native herbivores and their endoparasitoids. Performance of a specialist (*P. rapae*) and a generalist (*M. brassicae*) herbivore was compared on one cultivar and three wild populations of *B. oleracea* that differ in the expression of glucosinolates. In addition, performance of the two larval endoparasitoids *C. rubecula* and *M. mediator* was studied when developing in their respective hosts, *P. rapae* and *M. brassicae*.



development time, body mass) of herbivores, it can also affect that of parasitoids that develop inside the insect herbivores. High-quality plants may support high-quality hosts, which in return produce high-quality parasitoids. Alternatively, parasitoid development may be compromised indirectly by plant-derived compounds that are stored in the host. In part I the question addressed is: does food-plant quality affect development of a parasitoid in a similar way as it affects its host? What is the effect of a) food plant species, b) plant domestication and c) genetic variation in plant allelochemistry on herbivore and parasitoid performance?

In **chapter 3**, I evaluate food-plant quality of two different cultivated brassicaceous plant species, *Brassica oleracea* and *Sinapis alba*, for the specialist herbivore *P. xylostella* and its specialist larval endoparasitoid *D. semiclausum* (Fig. 1). Many studies examining plant-consumer interactions are performed at a single time of the year, which may ignore temporal variation in the expression of phenotypic plant traits that affect multitrophic interactions. In this study, the experiments have been repeated at three time points divided over the course of a growing season.

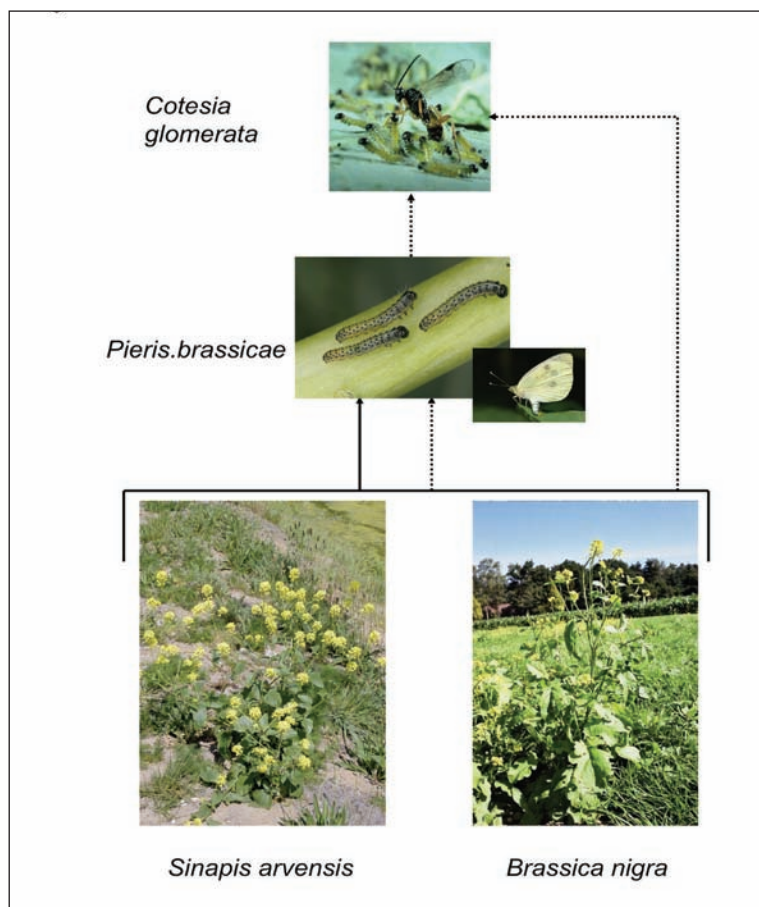
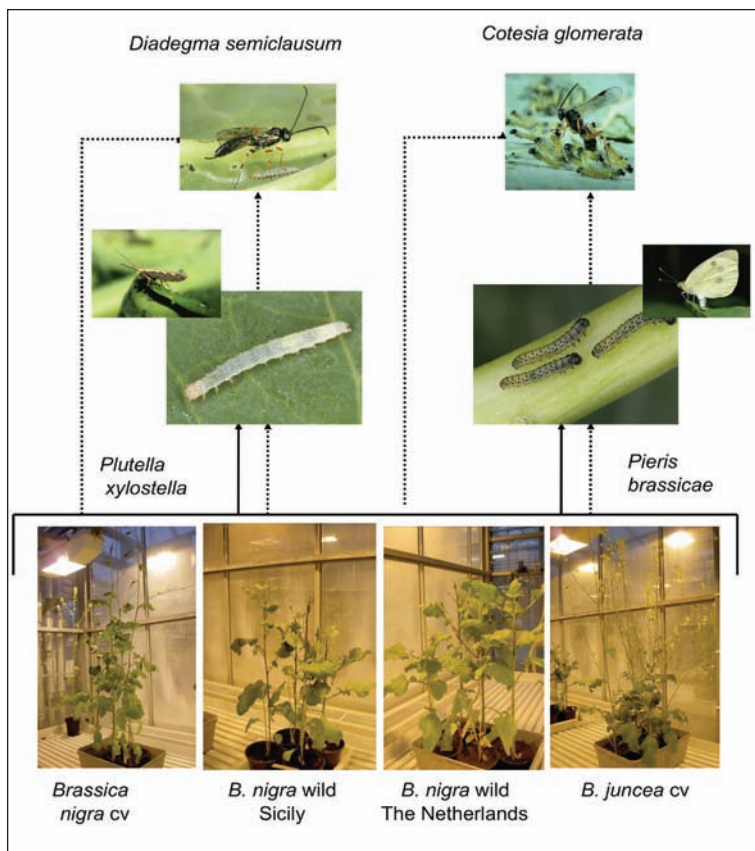


Figure 4: The effect of direct and indirect defences in two wild brassaceous plant species on a specialist herbivore and its gregarious endoparasitoid. Direct and indirect plant defences were investigated in two closely related wild brassaceous plant species, *B. nigra* and *S. arvensis*, which are both potential food plants for *P. brassicae* in The Netherlands. Food-plant quality was evaluated for both the herbivore and its larval endoparasitoid *C. glomerata*. In addition, flight-chamber experiments were conducted to reveal whether *C. glomerata* discriminates between the two plant species when damaged by its host.

Through the process of artificial selection, domesticated plants often contain reduced levels of phytochemicals, which may have concomitant effects on herbivore and parasitoid performance. This is the topic addressed in **chapter 4**. I compare insect performance on a cultivar and a wild population of *B. oleracea* as well as a feral *Brassica* population. Generalist herbivores are expected to be less strongly affected by food-plant phytochemicals than specialists adapted to feed on plants that contain specific chemicals such as glucosinolates in brassicaceous plant species. Here, I compare the performance on the three *Brassica* populations of several herbivore species differing in the degree of food-plant specialisation and two parasitoids differing in the degree of host specialisation (see Fig. 2).

In **chapter 5**, I address the performance of a specialist, *P. rapae*, and a generalist herbivore, *M. brassicae*, and their larval endoparasitoids, *C. rubecula* and *M. mediator*, respectively, on different wild *B. oleracea* populations (Fig. 3) that have been shown to differ in their glucosinolate levels (Mithen et al. 1995; Moyes et al. 2000).

Figure 5: Comparing direct and indirect plant defences in *Brassica nigra* populations that have been exposed to different selection pressures. Direct and indirect plant defences were investigated in two wild *B. nigra* (Brassicaceae) populations that originated from different geographical regions (The Netherlands and Sicily) as well as a cultivar, which has been subjected to artificial selection. A cultivar of *B. juncea*, a hybrid of *B. nigra* and *B. rapa*, was also included and was expected to differ more in plant chemistry than the *B. nigra* populations. Food-plant quality was evaluated for two specialist herbivore-parasitoid systems (*P. xylostella* - *D. semiclausum* and *P. brassicae* - *C. glomerata*). In addition, flight responses of the two parasitoid species were compared to plants of the different populations when infested by their respective hosts.



In Part II, potential conflicts between direct and indirect plant defences are addressed. Direct and indirect plant defences are usually studied independently of each other. However, these two plant defence strategies may not be mutually exclusive. For instance, plant secondary compounds have been demonstrated not only to affect insect herbivores but also the herbivore's natural enemies and a plant with high levels of direct defences may also have high levels of indirect defences. The specific question addressed in part II is: are direct and indirect defences compatible?

In **chapter 6**, I focus on both direct and indirect defences in two closely related wild brassicaceous plant species, *B. nigra* and *S. arvensis*, and their effects on a specialist herbivore, *P. brassicae*, and its gregarious endoparasitoid, *C. glomerata* (Fig. 4).

In **chapter 7** direct and indirect plant defences are compared among *B. nigra* populations that have been exposed to different selection pressures. Populations that grow in different geographical ranges are expected to be exposed to different abiotic and biotic selection pressures, which in turn may have concomitant effects on the performance of associated insect communities. I determined food-plant quality for two different

herbivore-parasitoid systems and I also measured parasitoid attraction to the different plant populations when damaged by hosts (Fig. 5).

Indirect defences, i.e., the attraction of natural enemies, are often studied in wind tunnels or Y-tube olfactometers. However, in nature, natural enemies of herbivores have to search for hosts in heterogeneous environments consisting of many different plant species. In part III (**chapter 8**), I investigate the foraging behaviour of the parasitoid, *D. semiclausum*, under semi-field conditions. Bukovinszky et al. (2005) demonstrated that in a Y-tube olfactometer *D. semiclausum* females do not discriminate between uninfested *S. alba* plants and *P. xylostella*-infested *B. oleracea* plants. Here, I investigate whether foraging behaviour of female *D. semiclausum* is affected by other brassicaceous and non-brassicaceous plant species in the vegetation in a more natural set-up than a Y-tube olfactometer. I determined whether plant composition and structural characteristics of a habitat affected the tendency of parasitoids to initiate foraging and to find a host-infested plant.

Finally, the results of this thesis are integratively discussed in the summarising discussion (**chapter 9**).

Acknowledgements

Pictures were kindly provided by Tibor Bukovinszky, Hans Smid and Renate Smallegange. I thank Marcel Dicke for constructive comments on draft versions of the manuscript.

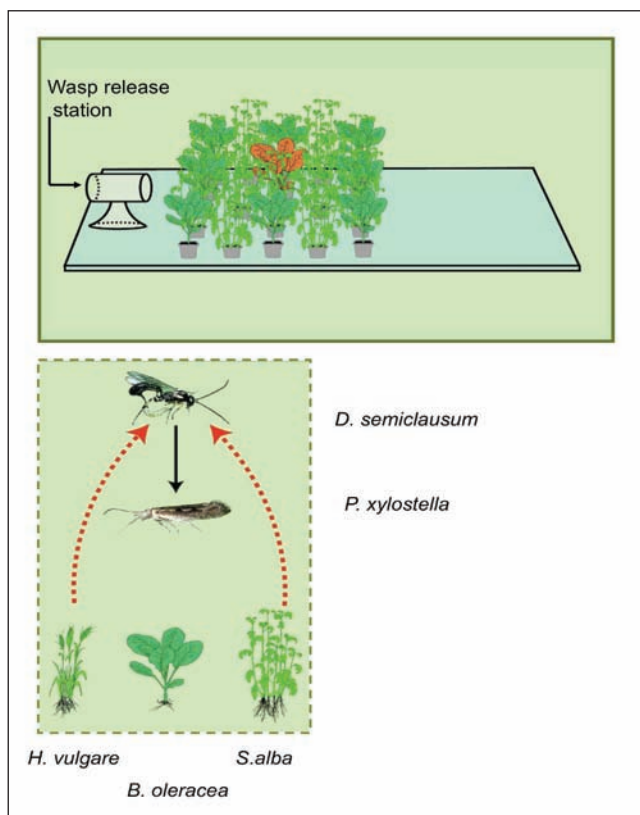
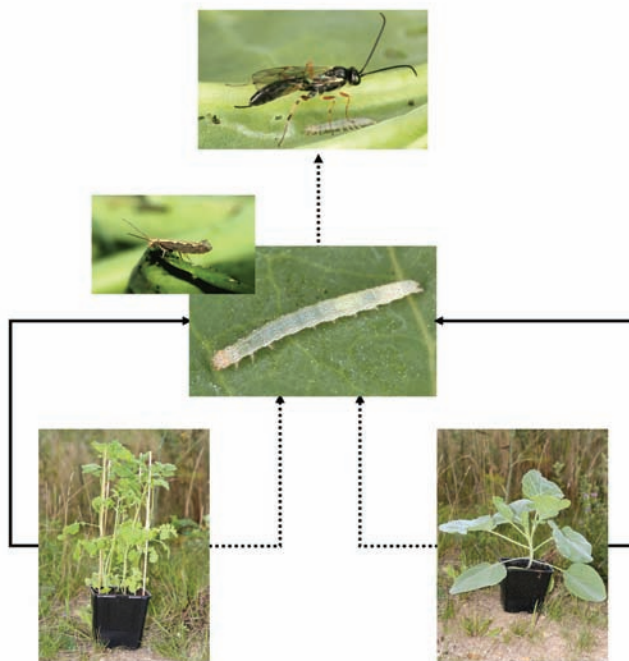


Figure 6: Reduced foraging efficiency of a parasitoid under habitat complexity: implications for population stability and species coexistence. Searching behaviour of the parasitoid, *Diadegma semiclausum*, was followed in different semifield set-ups, a low and high density monoculture of *Brassica oleracea* and two intercrops, *B. oleracea* with *Sinapis alba* (also a member of the Brassicaceae) and *B. oleracea* with *Hordeum vulgare* (Poaceae). Parasitoid foraging behaviour was also studied in set-ups in which the spatial arrangement (mixed versus row) or the height of the plants was varied. The drawing shows an intercrop of *B. oleracea* with *S. alba* in a mixed arrangement. The centre plant, a *P. xylostella*-infested *B. oleracea* plant, is the target plant for the parasitoid to find and is the same in all different set-ups.



**EFFECT OF PLANT
QUALITY ON
PERFORMANCE OF
HERBIVORES AND
THEIR PARASITOIDS**

PART ONE



3

Temporal changes affect plant chemistry and tritrophic interactions

by R. Gols, C.E. Raaijmakers, N.M. van Dam, M. Dicke,
T. Bukovinszky & Jeffrey A. Harvey

Published in *Basic and Applied Ecology* 8: 421-433 (2007)

In nature, individuals of short-lived plant species (e.g. annuals, biennials) may grow at different times during the growing season. These plants are therefore exposed to different season-related conditions such as light and temperature, which in turn may have consequences for primary and secondary chemistry of the plant. Despite this, many studies examining plant-consumer interactions are performed in single replicates, which may thus ignore temporal variation in the expression of phenotypic plant traits that affect multitrophic interactions. In the present study, we demonstrated that even under strictly controlled conditions in a greenhouse, secondary plant chemistry changes dramatically in plants growing at different times in a single year, i.e. July, August, and November. Glucosinolate (GS) contents in herbivore-damaged leaves of two different crucifer species, *Brassica oleracea* and *Sinapis alba* were higher in the August and November replicates than in the July replicate and GS concentrations were 10-25 times higher in *S. alba* than in *B. oleracea*. The development of a specialist herbivore, *Plutella xylostella*, also varied significantly over the three replicates. Larvae of *P. xylostella* that had fed upon either *S. alba* or *B. oleracea* attained the largest biomass and had the fastest development rate in the November replicate. Female *P. xylostella* moths grew larger on *S. alba* than on *B. oleracea*, whereas male biomass was not significantly affected by host-plant species. Plant species, but not season also affected performance of the endoparasitoid, *Diadegma semiclausum*. Similar to the performance of host females, parasitoid males developed faster and attained the highest biomass when attacking *P. xylostella* larvae feeding on *S. alba*. Most importantly, the performance of the herbivore and its parasitoid only appeared to partially conform to levels of GS in damaged leaves, indicating that there is a complex of factors involved in determining the effects of plant quality on higher trophic levels.

Keywords: *Brassica oleracea*, *Diadegma semiclausum*, direct defence, glucosinolates, host suitability, *Plutella xylostella*, *Sinapis alba*.

Introduction

Plants have evolved a range of physical (trichomes, spines, wax layers) and chemical (toxins, repellents and digestibility reducers) barriers to prevent or reduce insect feeding (reviewed by Karban and Myers 1989; Schoonhoven et al. 2005), which are collectively known as direct plant defences. Many insects have evolved counter-adaptations to circumvent these plant defences. They can avoid feeding on toxic plant parts or tissues (Dussourd 1993), and some insects can excrete or detoxify harmful plant compounds (e.g., Naumann et al. 2002; Ratzka et al. 2002). In addition, herbivores can sequester plant toxins and use them for their own defence (e.g., Müller et al. 2001). Many herbivores have evolved dietary specialisation and feed only on one or a few related plant species. The performance of specialised herbivores is often determined by the presence of specific chemicals in the host plant that stimulate feeding balanced against the negative effect of inhibitors (Gupta and Thorsteinson 1960b; Reed et al. 1989; van Loon and Schoonhoven 1999; Renwick 2002). Some secondary plant chemicals in potential host plants can negatively affect the development of specialised herbivorous insects (Zangerl and Berenbaum 1993; Stowe 1998; van Dam et al. 2000; Agrawal and Kurashige 2003; Steppuhn et al. 2004). Plant-defence chemicals are also transferred vertically through the food chain and can affect the performance of organisms in higher trophic levels (Campbell and Duffey 1981; Barbosa et al. 1991; Roth et al. 1997; Francis et al. 2001b; Harvey et al. 2003; Sznajder and Harvey 2003; Hartmann 2004).

Plant secondary chemistry is genetically determined, but is phenotypically plastic and varies in response to both biotic (e.g., pathogen infection and herbivore feeding, see Karban and Baldwin 1997; Agrawal 1999a) and abiotic (e.g., nutrient levels and light conditions, Takabayashi et al. 1994; Stout et al. 1998; van Dam et al. 2000; Gouinguene and Turlings 2002; Traw and Dawson 2002) factors. Seasonal effects on secondary chemistry of ephemeral plants, such as annuals, have received little attention in the literature (Feeny and Rosenberry 1982). However, many short-lived plants may germinate and grow at different times during the growing season; for example, *Sinapis arvensis* can be found between March and December in temperate latitudes (personal observation, J.A. Harvey). Abiotic factors, such as light and temperature, and the intensity of selection pressures from attackers, such as pathogens and herbivores, may also change dramatically during the year. Consequently, these changes may have significant effects on the primary and secondary chemistry of cohorts of annual plants that germinate at different times during the year. Different generations and/or guilds of herbivores associated with these plants may also be affected over the

seasons in which these plants grow. However, laboratory studies, carried out under controlled conditions, are often performed in single replicates and at any time of the year. They assume that the plant will exhibit standard phenotypic responses under these controlled conditions. Thus, whereas plant age is usually well defined in experimental studies, any possible effects of season in greenhouse-reared plants are usually ignored.

All plant species belonging to the Brassicaceae family are able to biosynthesise glucosinolates (β -thioglucocide-N-hydroxysulfates) (Fahey et al. 2001; Mithen 2001a). Glucosinolates (GS) are converted into biologically active thiocyanates, isothiocyanates, nitriles and oxazolidine-2-thiones by the enzyme myrosinase, which is stored and segregated from the GS substrate and is only released after wounding of the plant. GS and their breakdown products are considered to play an important defensive role against pathogens and insect herbivores. Members of the Brassicaceae vary considerably in GS quantity and composition (Rask et al. 2000; Fahey et al. 2001) and insect herbivores specialised on Brassicaceae differ in their performance on different members of this plant family (Fox et al. 1996; Ohsaki and Sato 1999; Sznajder and Harvey 2003). Differences in GS quantity and composition between crucifer species may thus contribute to differences in herbivore performance.

Plutella xylostella L. (Lepidoptera, Plutellidae) is a specialist herbivore that is known to feed on a number of species in the Brassicaceae. Adult females and larval stages use GS as oviposition and feeding stimulants, respectively (Gupta and Thorsteinson 1960a, b; Nayar and Thorsteinson 1963; Pivnick et al. 1994). However, an increase in GS concentration does not always positively correlate with larval performance (Li et al. 2000).

The aim of the present study is to evaluate host-plant quality over the course of a growing season for the herbivore *P. xylostella* feeding on two crucifer species that differ qualitatively and quantitatively in GS content, and to examine if there are also effects on the development of its solitary endoparasitoid, *Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae). The experiments were conducted over several months in a greenhouse with a controlled temperature regime but with seasonal (temporal) changes in light accessibility. Under these conditions we predicted that plant quality, based on levels of allelochemicals in plant tissues, would vary only slightly over the duration of the experiment. Because of this, we expected that the effects of marginal differences in plant defences on the herbivore and its endoparasitoid would be negligible but that insect performance would still be correlated with the levels of GS.

Our results reveal that even under fairly controlled temperature conditions, there are dramatic temporal (seasonal)

shifts in foliar levels of plant allelochemicals. However, herbivore and parasitoid performance was not strictly correlated with plant defences revealing that other factors are involved that affect their development. We argue that the variation and expression of abiotic and biotic stresses in the field are likely to be far larger than in the laboratory, and that there may be significant consequences for the biology and ecology of generalist and specialist consumers over several trophic levels.

Materials and methods

Insects

Larval and adult *P. xylostella* were obtained from cultures maintained in the laboratory on *B. oleracea* plants (cv. Cyrus). The parasitoid, *D. semiclausum* was collected from Brussels sprout fields in the vicinity of Wageningen (The Netherlands) and was reared on larvae of the diamondback moth, *P. xylostella*. A new generation of *D. semiclausum* was started weekly. About 40 wasps in a 50:50 male-to-female sex ratio were added to a cage with two *B. oleracea* plants heavily infested with second and third instar *P. xylostella* larvae. Extra food plants were added regularly for the caterpillars, as well as honey and water for the parasitoids. Two weeks after introduction of the wasps, all plant material was removed and cocoons were collected and transferred to a clean insect cage (37 x 40 x 30 cm) without plants. Emerging wasps were collected daily and used either for parasitoid rearing or experiments. The cultures of *P. xylostella* and *D. semiclausum* were maintained in a climate room, 16L:8D photoperiod at 21 ± 2 °C and a relative humidity of 70%. Wasps were collected over a three week period from three different generations and kept collectively in a cage (42 x 22 x 22 cm) at 12 ± 2 °C to extend their longevity. Wasps were provided *ad libitum* with water and honey, which was administered to the gauze wall of the cage.

Plants

In cultivated *B. oleracea* L. plants, the most dominant GS is the indole GS glucobrassicin, whereas in *Sinapis alba* L. the aromatic sinalbin is the major GS component (Reed et al. 1989; Hopkins et al. 1998; Renwick and Lopez 1999; Francis et al. 2001b).

Brassica oleracea gemmifera cv. Cyrus (Brussels sprouts) and *S. alba* cv. Carneval (white mustard) were grown in a greenhouse (25 ± 5 °C, 40-80% r.h. and a photoperiod of at least 16 h). If the light dropped below 500 $\mu\text{mol photons/m}^2/\text{s}$ during the 16-h photoperiod, supplementary illumination was applied by high-pressure mercury lamps. Seeds were sown individually in pots (11 x 11 x 11 cm) filled with potting soil ("Lentse potgrond" no. 4, Lent, The Netherlands). When plants were used for oviposition by *P. xylostella*, mustard plants were 3-4 weeks old and Brussels

sprout plants were 5-6 weeks old. Brussels sprout plants were sown on June 4, July 7 and October 1, and sowing dates for mustard were June 18, July 18 and October 13, 2002.

Bioassay

We monitored the development of parasitised and unparasitised *P. xylostella* larvae that were feeding on either *B. oleracea* or *S. alba*. To investigate the effect of instar at parasitism on wasp development, host larvae were parasitised by *D. semiclausum* in either the second (L2) or fourth (L4) larval instar (see below). To obtain larval stages of *P. xylostella* on the two different plants species, two plants of each species were placed in separate cages per plant species with c. 100 *P. xylostella* moths in a 50:50 female-to-male ratio. After a 24-h oviposition period, the moths were removed and the plants were left in the cages for the development of the eggs into larvae. Extra plants were added to the cages to sustain larval growth. When the larvae reached the second instar, a cohort of 70-100 unparasitised caterpillars was transferred to new plants and a second cohort of 100-110 individuals was parasitised by *D. semiclausum*. An equal number of *P. xylostella* larvae was parasitised when the unparasitised larvae reached the fourth instar.

For parasitism, a vial with a single mated female wasp was placed over a leaf with a single host larva. After the larva was seen to be parasitised, it was transferred to its respective experimental host plant with a fine paintbrush. Each female wasp was used to parasitise up to six individual hosts. Parasitised larvae were evenly distributed over undamaged host plants, 10 plants for mustard and 9 plants for Brussels sprouts (Brussels sprout plants had about 10% more biomass than mustard plants). Each individual plant received 10-12 larvae. Plants of the same species and the same treatment (parasitised as L2, as L4, or unparasitised) were placed together in a cage, (60 x 60 x 100 cm). The larvae were allowed to move freely from one plant to another within their respective cage. When the larvae reached the fourth instar, pieces of corrugated cardboard (5 x 20 cm) were placed on top of the plants as the larvae tend to pupate in sheltered places. This prevented the larvae pupating directly on the cage walls. Pupae were carefully cut out from the cardboard or removed from the leaves on which they had pupated and transferred individually to labeled glass vials. When adults emerged, either as moths or wasps, the egg-to-adult development time was recorded. Vials were checked every 2 h, but development time was recorded in full days since the exact time of oviposition had not been recorded. Adults were frozen and dried for 72 h in an oven at 80°C and weighed on a Cahn C-33 microbalance (Cahn instruments, USA). To investigate the effect of season, the same experiment was conducted at three different times during the growing season. The first trial was performed in

July, the second in August and the third in November.

All experiments were conducted in a climate room, 16L:8D photoperiod at 21 ± 2 °C and a relative humidity of 70%. Mercury lamps hanging above the cages provided additional light during the photoperiod (40 ± 10 $\mu\text{mol photons/m}^2/\text{s}$).

Glucosinolate analysis

In each replicate experiment, leaf samples for GS analysis were taken from plants damaged by parasitised hosts. The larvae had been feeding on the plants for 4-8 days and leaves were sampled when the larvae moved away from the plant or had already pupated. Faeces and pupae were carefully removed from the leaves. Leaves were taken from position 4-6 on *B. oleracea* and position 4-7 on *S. alba* (leaf 1 is the youngest leaf and leaf 4 is the first fully developed leaf) and 4-5 leaves were sampled per leaf position. One leaf was taken per plant and all sampled leaves had only moderate traces of feeding damage. Leaves were cut off using a razor blade and immediately stored at -80°C. Leaves were analysed individually. Samples were freeze dried and ground to a fine powder with a mortar and pestle. Aliquots of 50 mg for *S. alba* and 100 mg for *B. oleracea* were weighed in 15-ml centrifuge tubes. GS were extracted and purified as in van Dam et al. (2004). GS were separated on a reverse phase C-18 column (Alltima C-18, 3 μm , 150 x 4.6 mm, Alltech, Deerfield, IL, USA) on HPLC (Dionex, Sunnyvale, CA, USA) with an acetonitrile water gradient. Detection was performed with a Dionex PDA-100 Photodiode array detector set to scan from 200 to 350 nm. For quantification, sinigrin (Sigma, St. Louis, MO, USA) was used as an external standard. Peaks were integrated at 229 nm for which standard response factors have been defined (EC, 1990). The different GS were identified based on their retention times and UV spectra compared to those of pure compounds (sinigrin, Sigma, St. Louis, MO, USA), glucotropaeolin, sinalbin, glucobrassicin (kindly provided by M. Reichelt, MPI for Chemical Ecology, Jena, Germany), or compared to a certified oil seed reference (EC Community Bureau of Reference BCR-367R, Fluka, Buchs, Switzerland)

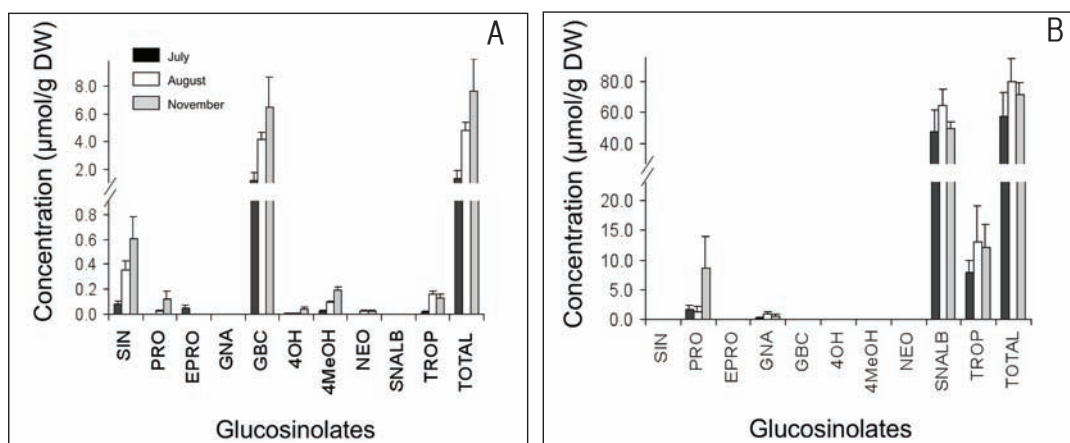
Statistical analysis

Data on adult dry mass and development time of unparasitised *P. xylostella* were analysed using 3-way ANOVA with plant species, sex and replicate and their interactions as factors. For the parasitoid data, males and females were analysed separately with host instar at parasitisation, plant species and replicate as factors. The Tukey HSD method was used for multiple comparisons of means. To examine GS content two-way ANOVA were used with replicate and leaf position as factors.

Results

Glucosinolate analysis

The two plant species differed qualitatively and quantitatively in GS content (Fig. 1). In *B. oleracea* eight different GS were detected: three alkenyl GS (progoitrin, epiprogoitrin, and sinigrin), four indole GS (glucobrassicin, 4-hydroxy glucobrassicin, 4-methoxy glucobrassicin, and neoglucobrassicin), and the aromatic glucotropaeolin. The most dominant GS in *B. oleracea* was glucobrassicin. On average 90% of the GS dry weight mass consisted of this compound. In *S. alba* four different GS were found, two alkenyl compounds, i.e. progoitrin and gluconapin, and two aromatic GS, i.e. (gluco)sinalbin and glucotropaeolin. Sinalbin was the dominant GS representing on average more than 70% of the total GS content.



GS concentrations were significantly different for the three different moments in the season for all GS in both plant species (Table 1). GS contents also differed depending on the position of the leaf on the plant, with the youngest leaf containing the highest concentration of GS. In *B. oleracea* total GS levels were 1.5-4.5 fold higher in the 4th leaf compared to the 6th leaf from the top. In *S. alba*, the total GS concentration was 1.5-2.7 fold higher in the 4th compared to the 7th leaf from the top. In *B. oleracea*, a significant effect was found for leaf position and GS content for sinigrin, 4-methoxy glucobrassicin and glucobrassicin and in *S. alba* for gluconapin and sinalbin.

Total GS contents were also different for the two plant species (Fig. 1). In the July replicate, the total GS concentration in leaves at position 4 from the top was 40 times higher in *S. alba* than in *B. oleracea*. In *B. oleracea*, the GS concentration in leaves at position 4 from the top was 5.5 times higher in November than in July. The change in GS concentration in *S. alba* during the

Figure 1: GS concentrations (mean + SE) in *B. oleracea* (A) and *S. alba* (B) in leaves sampled from the plant at position 4 from the top after *P. xylostella* had completed larval development (PRO: progoitrin; EPRO: epiprogoitrin; SIN: sinigrin; 4OH: 4-hydroxy glucobrassicin; 4MeOH: 4-methoxy glucobrassicin; TROP: glucotropaeolin; NEO: neoglucobrassicin; GBC: glucobrassicin; GNA: gluconapin; SNALB: sinalbin). Leaves were sampled from plants that had been used in experiments in July, August and November. Four to five plants of each plant species were sampled in each of the three experiments.

season was less dramatic. The highest concentrations (in leaves at position 4 from the top) were found in August and November and were respectively 1.4 and 1.3 times higher than in July.

Table 1: Two-way ANOVA's on glucosinolate content with replicate and leaf position as main factors

<i>B. oleracea</i> Glucosinolate	Replicate		Leaf position	
	$F_{2,37}$ -value	P-value	$F_{2,37}$ -value	P-value
Progoitrin	6.55	0.004	2.68	0.082
Epiprogoitrin	4.91	0.01	1.38	0.26
Sinigrin	9.11<0.001	4.75	0.01	
4-OH glucobrassicin	26.27	<0.001	0.88	0.42
4-MeOH glucobrassicin	49.64	<0.001	5.75	0.007
Glucobrassicin	13.50	<0.001	3.99	0.03
Neoglucobrassicin	12.21	<0.001	2.17	0.13
Glucotropaeolin	5.07	<0.001	0.88	0.42
Total	15.80	<0.001	4.84	0.01
<i>S. alba</i>				
Glucosinolate	$F_{2,46}$ -value	P-value	$F_{3,46}$ -value	P-value
Progoitrin	4.90	0.011	1.96	0.11
Gluconapin	3.36	0.04	3.57	0.01
Sinabin	8.37<0.001	3.02	0.03	
Glucotropaeolin	7.21	0.002	1.52	0.21
Total	10.74	<0.001	4.30	0.005

Effect of host-plant species on the herbivore *P. xylostella*

Female *P. xylostella* readily accepted both *S. alba* and *B. oleracea* as host plants for oviposition. More than 95 % of the monitored unparasitised larvae developed into adult moths. All factors included in the model had a significant effect on adult biomass (see Table 2). Adult females grew on average 1.6 and 1.8 times heavier than males (measured as dry mass) on cabbage and mustard, respectively. The difference in this ratio between the two host plants was consistent over the three replicates. Female moths were heavier on *S. alba* (mean dry mass $\pm$ SE: 1.82 ± 0.02 mg), than on *B. oleracea* (1.71 ± 0.04 mg), (Fig. 2). Moths attained the highest biomass in the November replicate on both plant species (Fig. 2).

There was a significant effect of replicate and a marginal effect of sex on egg-to-adult development time of the moths (see Table 2). Moths developed fastest in the November replicate (Fig. 3). Females took on average 17.0 ± 0.05 (mean $\pm$ SE) days to develop from egg to adult and males took 17.2 ± 0.07 days. There was also a significant interaction between plant species and replicate, and between plant and sex (see Table 2). Development of females was faster on *S. alba* than on *B. oleracea* (Fig. 3). The fastest development was found for males on *B. oleracea* in the November trial (Fig. 3).

Effect of host instar and plant species on the parasitoid *D. semiclausum*

On average 83 prepupae or pupae were recovered per treatment out of the 100-110 parasitised larvae that were put on the plants

Factors	Adult dry mass		Development time	
	F-value(d.f.)	P-value	F-value	P-value
<i>Plutella xylostella</i>				
Total model	105 (9,427)	<0.001	7.6 (9,425)	<0.001
Sex	859 (1,427)	<0.001	3.7 (1,425)	0.056
Plant species	10 (1,427)	0.002	0.01 (1,425)	0.91
Replicate	20 (2,427)	<0.001	17 (2,425)	<0.001
Sex x plant species	6.3 (1,427)	0.01	15 (1,425)	<0.001
Sex x replicate	3.5 (2,427)	0.03	1.4 (2,425)	0.23
Plant species x replicate	8.5 (2,427)	<0.001	7.1 (2,425)	<0.001
<i>Diadegma semiclausum</i> males				
Total model	5.8 (9,621)	<0.001	11 (9,620)	<0.001
Instar	17 (1,621)	<0.001	38 (1,620)	<0.001
Plant species	17 (1,621)	<0.001	6.0 (1,620)	0.01
Replicate	0.25 (2,621)	0.78	2.8 (2,620)	0.06
Instar x plant	3.7 (1,621)	0.054	12 (1,620)	<0.001
Instar x replicate	2.5 (2,621)	0.08	16 (2,620)	<0.001
Plant species x replicate	5.5 (2,621)	<0.004	7.3 (2,620)	<0.001
<i>Diadegma semiclausum</i> females				
Total model	1.9 (9,97)	0.059	3.7 (9,97)	<0.001
Instar			11 (1,97)	0.002
Plant species			3.6 (1,97)	0.06
Replicate			3.3 (2,97)	0.04
Instar x plant species			2.5 (1,97)	0.11
Instar x replicate			3.4 (2,97)	0.04
Plant species x replicate		2.9 (2,97)		0.06

Table 2: Three-way ANOVA's on egg-to-adult development time and adult body mass of moth and parasitoid males and females

at the start of the experiment (Table 3). About 77% of these pupae produced adult *D. semiclausum* and about 8% produced adult *P. xylostella* (Table 3). A few dead larvae were found, but most of the larvae that had not pupated could not be retrieved from the plants.

Since *D. semiclausum* is subject to complementary sex determination (Butcher et al. 2000), the number of females emerging from parasitised larvae was low, 106 females compared to 626 males. Higher sex ratios were obtained from *P. xylostella* larvae that were parasitised as L4, 17.5% females compared to 9% females from L2 larvae. Females grew larger than males (ANOVA, $F_{1,734} = 75$, $P < 0.001$). Mean dry mass of females ($\pm$ SE) was 0.761 ± 0.014 mg compared to 0.588 ± 0.009 mg for males. There was no significant effect of sex on development time (ANOVA; $F_{1,733} = 0.24$, $P = 0.63$). Wasps took on average 15.4 ± 0.02 days to develop from egg to adult. Since the number of females in some of the treatments was very low (see Table 1), the effect of instar at parasitisation, host plant species and replicate was analyzed separately for males and females.

Males: Wasps were significantly larger and egg-to-adult development time was significantly shorter when hosts were parasitised as L4 compared to L2 (see Table 2, Fig. 4A and 5A). Wasps also grew larger and developed faster when the host was feeding on *S. alba* compared to *B. oleracea*. The longest development time and smallest wasps were recorded for wasps emerging from hosts that were parasitised as L2 that had fed on *B. oleracea* in the July and August replicate.

Figure 2: Dry weight (mean + SE) of female and male *P. xylostella* moths that had been reared on *S. alba* or *B. oleracea*. Experiments have been conducted in July, August and November. Bars with the same letter are not significantly different (Tukey HSD test for multiple comparisons among means with $\alpha = 0.05$). Numbers between brackets denote number of individuals (*n*).



Figure 3: Egg-to-adult development time (mean + SE) of female and male *P. xylostella* moths that had been reared on *S. alba* or *B. oleracea*. Experiments have been conducted in July, August and November. Bars with the same letter are not significantly different (Tukey HSD test for multiple comparisons among means with $\alpha = 0.05$). Numbers of individuals (*n*) are the same as in Fig. 1.



Females: As in males, females developed significantly faster when the hosts were parasitised as L4 larvae (Table 2, Fig. 5B). The shortest development time was found for female wasps developing from hosts that had fed on *B. oleracea* and were parasitised as L4 larvae. Biomass was not influenced by any of the factors included in the model (Fig. 4B, Table 2).

Discussion

An overview of the empirical literature reveals many studies reporting that differences in plant chemistry, particularly secondary compounds can have a profound effect on the biology, physiology

Treatment	Pupae total #	Moth female	Wasp male	Wasp	Dead
July					
<i>B. oleracea</i> - L2	72	17	5	36	14
<i>B. oleracea</i> - L4	78	2	20	44	12
<i>S. alba</i> - L2	75	6	8	44	17
<i>S. alba</i> - L4	77	0	12	51	14
August					
<i>B. oleracea</i> - L2	83	13	7	56	7
<i>B. oleracea</i> - L4	85	1	7	52	25
<i>S. alba</i> - L2	61	4	2	47	8
<i>S. alba</i> - L4	95	10	12	61	12
November					
<i>B. oleracea</i> - L2	89	5	7	45	7
<i>B. oleracea</i> - L4	96	6	14	71	5
<i>S. alba</i> - L2	87	5	8	63	11
<i>S. alba</i> - L4	93	9	6	56	22

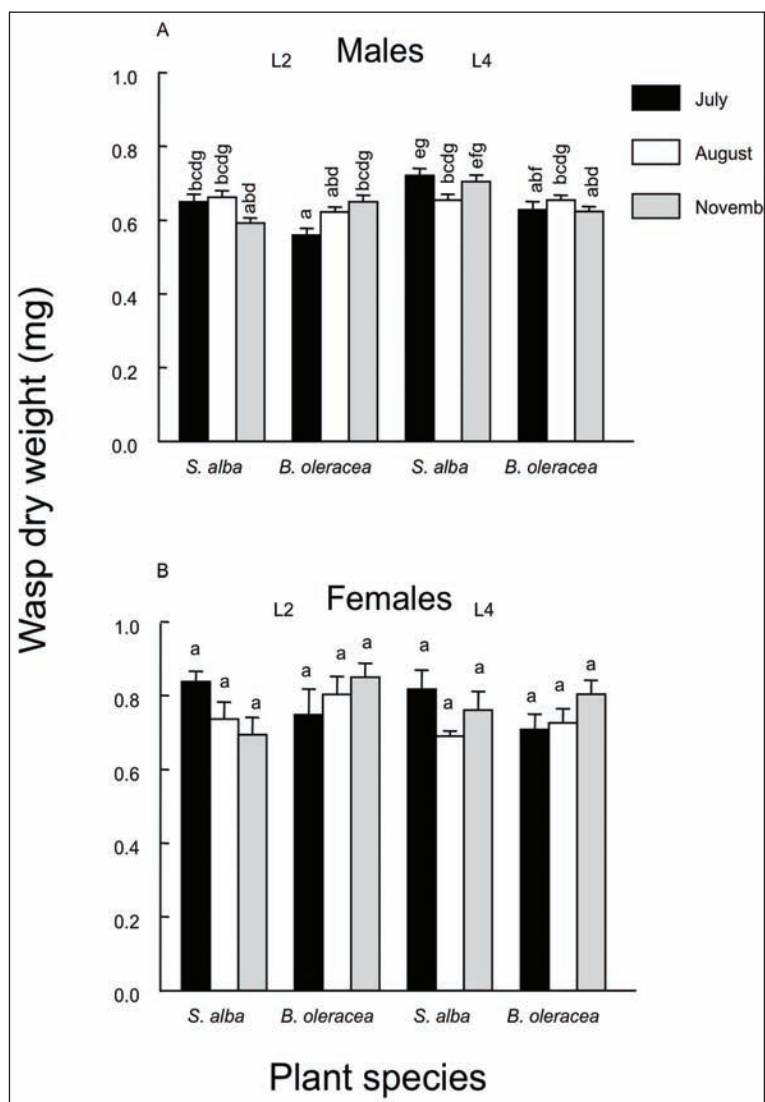
Table 3. Recovery (total number of pupae) and fate of the pupae of which the larvae had fed on *B. oleracea* or *S. alba* and were parasitised by *D. semiclausum* as second (L2) or fourth instar (L4). Pupae developed into *P. xylostella* moths, *D. semiclausum* wasps (female or male) or did not emerge (dead)

and ecology of associated consumers, i.e. herbivores and their natural enemies (e.g., Campbell and Duffey 1981; Barbosa et al. 1991; Roth et al. 1997; Francis et al. 2001b; Harvey et al. 2003; Sznajder and Harvey 2003; Hartmann 2004). However, the results of most of these studies have been derived from single experiments that were carried out at a specific time during the year. In this study we have shown that, in two closely related plant species, there are profound (and species-specific) temporal changes in plant secondary chemistry. These changes occurred even when abiotic conditions (temperature, photoperiod) were primarily controlled. Moreover, the performance of *P. xylostella* and its parasitoid *D. semiclausum* also varied between replicates, although in both plants it did not appear to be correlated with GS levels in leaf tissues.

In many plant species, such as arable weeds, different populations may germinate and grow at different times of the year, which is true in many species of the Brassicaceae. Our results thus imply that under natural conditions, seasonal shifts in abiotic conditions, such as light availability and intensity, as well as temperature, may exert even more dramatic effects on plant ecophysiology and that these will have variable effects on higher trophic levels intimately associated with the plant. Earlier studies have reported that temporal changes in plant quality affect the performance of bi- or multi-voltine herbivores and their natural enemies. This has been demonstrated in consumers associated with long-lived plants such as trees where changes in the chemistry and toughness of leaves during the growing season are well documented (Feeny 1970; Riipi et al. 2002).

Chemical analyses revealed that GS concentrations not only varied between plant species but also over the different

Figure 4: Dry weight (mean + SE) of male (A) and female (B) *D. semiclausum* wasps that had been reared on *S. alba* or *B. oleracea* and were parasitised as L2 (first six bars) or L4 (last six bars). Experiments have been conducted in July, August and November. Bars with the same letter are not significantly different (Tukey HSD test for multiple comparisons among means with $\alpha = 0.05$). Number of individuals (n) in each treatment can be found in Table 1.



replicates that were carried out at different times during the growing season. Plants used in the different replicates were of the same size, but plants in the November replicate took 1 week longer to reach the same size as the plants grown in July and August. The highest GS concentrations were found in the November replicate for *B. oleracea* and in the August and November replicates for *S. alba*. Feeny and Rosenberry (1982) reported that GS concentrations in the annual, *B. nigra*, declined during the growing season. However, in that study the age of the *Brassica nigra* plants confounded the results, whereas the present study shows that within-plant GS contents declined with leaf age, i.e. older leaves have lower GS concentrations than younger leaves. Agerbirk et al. (2003) showed that GS concentration in natural populations of the crucifer *Barbarea vulgaris* increase

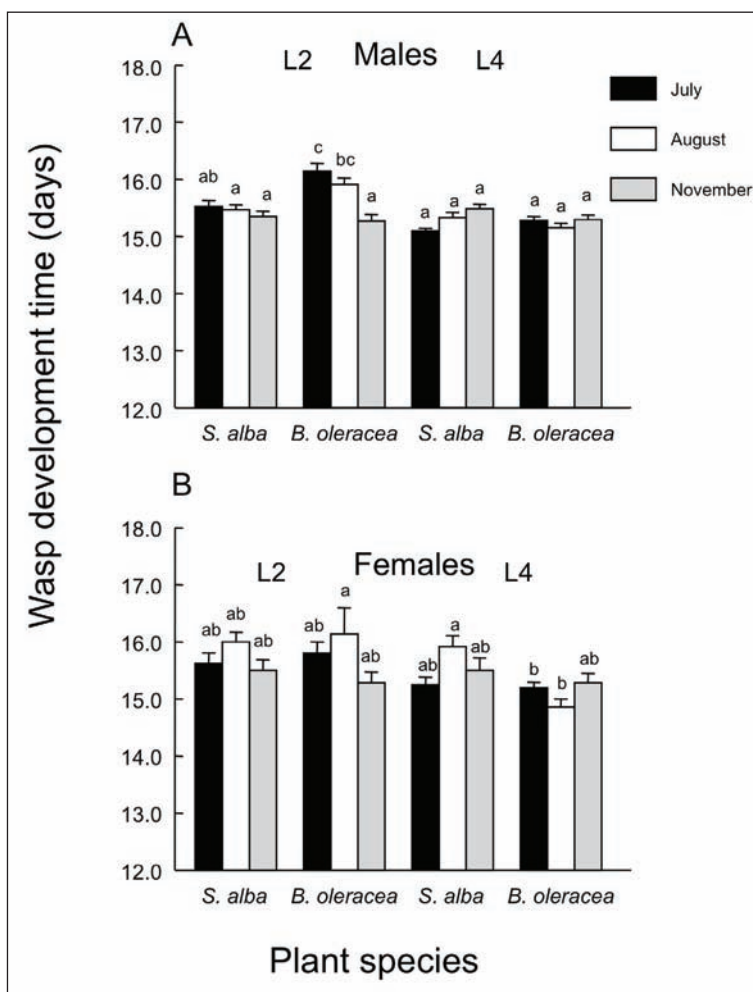
from August to November. Furthermore, season and myrosinase activity in cultivars of *B. oleracea* were significantly correlated in a study by Charron et al. (2005) with higher enzyme activity in the fall.

Chemical analyses not only revealed quantitative differences but also qualitative differences between GS in leaf tissue of *B. oleracea* and *S. alba*. Total GS concentrations were ten to forty times higher in *S. alba* than in *B. oleracea*, and GS composition in the two plant species was very different, with sinalbin being the dominant GS in *S. alba* and glucobrassicin in *B. oleracea* (Hopkins et al. 1998; Moyes et al. 2000; Francis et al. 2001b). Cultivated plant species often contain lower concentrations of secondary plant compounds than their wild relatives (Evans 1993). Artificial selection in *B. oleracea* is aimed at the production of edible structures, where low levels of bitter-tasting GS are desired (Brussels sprout, cabbage, cauliflower, etc.). By contrast, seeds of *S. alba* are used as a condiment and usually contain high levels of GS. High levels of GS in seeds are not necessarily correlated with high levels of these compounds in other plant tissues (Rosa 1999), although in the present study, much higher foliar concentrations of GS were found in *S. alba* than in *B. oleracea*. Furthermore, GS levels in the cultivated *B. oleracea* used here are much lower than GS levels in wild populations of *B. oleracea* (Moyes et al. 2000). Differences in directional selection in *B. oleracea* and *S. alba* may have resulted in the observed differences in total GS contents.

Sinapis alba appeared to be a qualitatively better host plant for the development of *P. xylostella* than *B. oleracea*. Male and female moths were differentially affected by the host plant upon which they had fed. Female moths attained a higher biomass on *S. alba* than on *B. oleracea*, whereas males were not affected by host plant species. Sexes may differ in food requirements and these differences already occur in the immature stage when resources are acquired for future needs (Slansky 1993). Since there was no difference in development time between the sexes, female moths must have a higher rate of consumption or else are more efficient in food utilisation than male moths. A study by Raps and Vidal (1998) similarly reported that plants inoculated with an endophytic fungus affected the development of female *P. xylostella* more than males. Females feeding on endophyte-infested leaves exhibited a reduced conversion efficiency of ingested food and increased their relative consumption rates (Raps and Vidal 1998).

Larval stages of *P. xylostella* use GS as feeding stimulants, but not all GS are equally effective (Nayar and Thorsteinson 1963) and an increase in GS concentration does not always positively correlate with larval performance (Li et al. 2000). Progoitrin and to a lesser extent glucotropaeolin are strong feeding stimulants for *P. xylostella* larvae, whereas gluconapin is toxic at higher

Figure 5: Egg-to-adult development time (mean + SE) of male (A) and female (B) *D. semiclausum* wasps that had been reared on *S. alba* or *B. oleracea* and were parasitised as L2 (first six bars) or L4 (last six bars). Experiments have been conducted in July, August and November. Bars with the same letter are not significantly different (Tukey HSD test for multiple comparisons among means with $\alpha = 0.05$). Number of individuals (n) in each treatment can be found in Table 1.



concentrations (Nayar and Thorsteinson 1963). Progoitrin and glucotropaeolin are found in much higher concentrations in *S. alba* than in *B. oleracea* and progoitrin levels increased over the growing season in both plant species. On the other hand gluconapin was only detected in *S. alba*. Thus, performance of the herbivore only partially conformed to the levels of GS found in damaged leaves. Levels of some GS change after feeding damage by herbivores (Bartlett et al. 1999). In this study, leaf sampling for GS occurred after the larvae had stopped feeding and therefore reflects only the final chemical composition to which the herbivores have been exposed. This could also explain why we did not find a relationship with herbivore performance and plant chemistry. The presence of feeding stimulants together with nutrients such as proteins and carbohydrates, as well as digestibility reducers and deterrents determines food plant quality. It is very likely that not only GS, but also the primary plant metabolites, as well as plant compounds that negatively

influence insect growth varied in the three replicates. These may have also influenced performance of the herbivore in the three replicates.

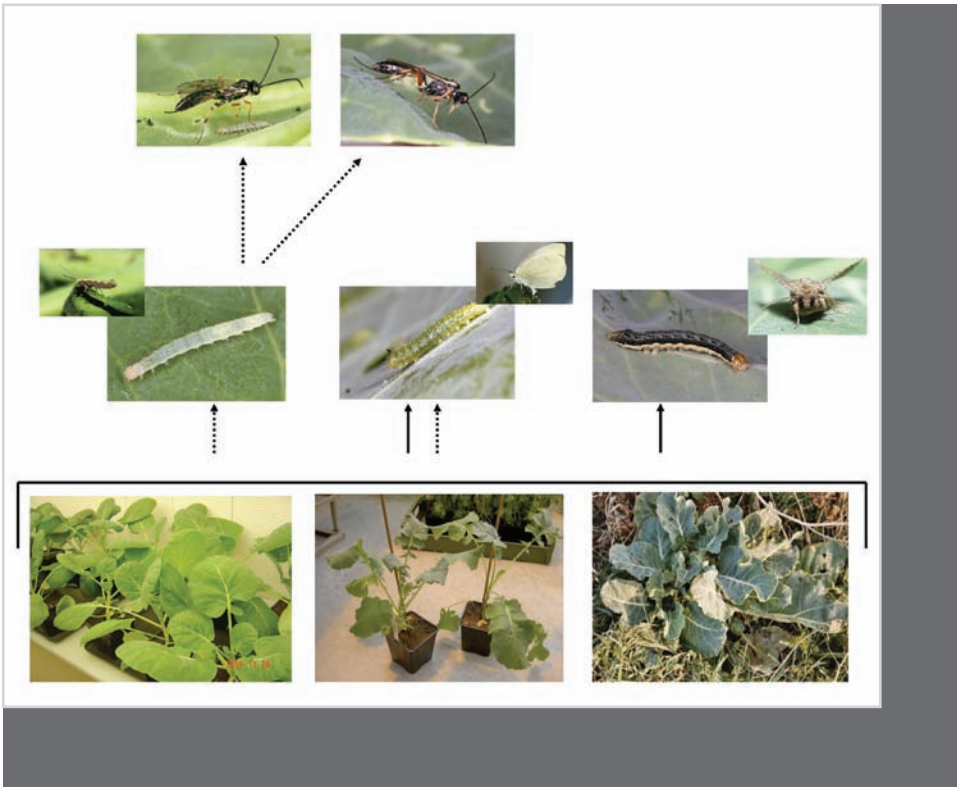
Host plant species not only affected herbivore performance but also the development of the endoparasitoid, *D. semiclausum*. Parasitoid males developed faster and attained the highest biomass when attacking hosts feeding on *S. alba*. However, temporally replicated experiments did not result in significant effects on parasitoid biomass nor on development time as had been demonstrated in the herbivore. *Plutella xylostella* uses the enzyme, GS sulfatase, which can desulfate a broad range of GS making them unsuitable substrates for myrosinase activity, and thereby circumventing the production of toxic hydrolysis products such as nitriles, thiocyanates or isothiocyanates (Ratzka et al. 2002). The host provides all of the resources necessary for parasitoid development and the quality of the host can be affected by secondary plant compounds ingested by the herbivore (Campbell and Duffey 1981; Barbosa et al. 1991; Roth et al. 1997; Harvey et al. 2003; Sznajder and Harvey 2003). However, detoxification of the GS by the host may dilute the effect of these compounds on parasitoid growth, although it has been shown that host-plant species can influence not only the third but the fourth trophic level as well (Harvey et al. 2003). On the other hand, specialised parasitoids, like their hosts, may have also evolved mechanisms to deal with secondary plant compounds.

The effect of host instar at parasitism (L2 or L4) had a strong effect on the development of *D. semiclausum*. On both plant species, wasps developed faster and attained the highest biomass when the larvae were parasitised as L4. *Diadegma semiclausum* can successfully develop in all four larval stages of *P. xylostella*, but the offspring sex ratio (percentage males) decreases when the host is parasitised as L4 (Yang et al. 1993). In this study, more females emerged from larvae parasitised as L4. Many parasitoids lay unfertilised eggs, which develop into males, in hosts of low quality (Godfray 1994). Our results indicate that L4 *P. xylostella* larvae are qualitatively better hosts for *D. semiclausum* than L2 larvae. Females of the parasitoid as well as females of the moth are larger than their male conspecifics. Therefore, it would be interesting to find out when during the development of the host caterpillar sexual size dimorphism occurs and whether an ovipositing wasp is able to detect sex in the host caterpillar (see also Gunasena et al., 1989).

A recent study showed that *D. semiclausum* prefers the odours emitted by *S. alba* to odours emitted by *B. oleracea* (Bukovinszky et al. 2005). Moreover, when behaviour of the parasitoid was followed in a semi-field set-up in a greenhouse with both plant species and only one *Plutella*-infested *B. oleracea* plant, *D. semiclausum* females were clearly more attracted to the *S. alba* plants even though there were no hosts present on

this plant (Gols et al. 2005). The present study shows that *D. semiclausum* is attracted to the plant species that is qualitatively a better plant for its host, *P. xylostella*, and to a lesser extent for its own development. It would be interesting to investigate whether *P. xylostella* adult females prefer to oviposit and larvae prefer to feed on those plant species with the highest nutritional quality. Field studies carried out during the growing season should reveal how changes in plant chemistry affect oviposition behaviour of adult females.

In summary, our study has revealed that there may be significant temporal changes in the secondary chemistry of short-lived plants in which different populations/cohorts grow at various times during the year even under fairly controlled conditions in a greenhouse compartment. In nature, we might expect even more significant seasonal changes in plant chemistry, because this would incorporate a range of other abiotic and biotic selection pressures such as day length, light intensity, temperature and temporal variation in the risk of attack from herbivores and pathogens. Importantly, conspecifics of annual plants that grow at different times during the year will be exposed to different light intensities and different day lengths, which will very likely affect secondary plant chemistry much stronger than plants that are grown at different times during the year under controlled greenhouse conditions. Different generations of multivoltine insect herbivores may therefore feed on individual plants whose chemistry varies dramatically, and by association their parasitoids may also experience considerable season-related differences in host plant quality, which may affect their development. Longer-term studies are thus required in order to better understand how potentially dynamic changes in plant quality affect a range of multitrophic interactions and community-level processes.



4

Performance of generalist and specialist herbivores and their endoparasitoids differs on cultivated and wild *Brassica* populations

by R. Gols, T. Bukovinszky, N.M. van Dam, M. Dicke,
J.M. Bullock & J.A. Harvey

Published in *Journal of Chemical Ecology* 34:132-143 (2008)

Through artificial selection, domesticated plants often contain modified levels of primary and secondary metabolites compared to their wild progenitors. It is hypothesised that the changed chemistry of cultivated plants will affect the performance of insects associated with these plants. In this paper, the development of several specialist and generalist herbivores and their endoparasitoids were compared when reared on a wild and cultivated population of cabbage, *Brassica oleracea*, and a recently established feral *Brassica* species. Irrespective of insect species or the degree of dietary specialisation, herbivores and parasitoids developed most poorly on the wild population. For the specialists, plant population influenced only development time and adult body mass, whereas for the generalists, plant populations also affected egg-to-adult survival. Two parasitoid species, a generalist (*Diadegma fenestrale*) and a specialist (*D. semiclausum*), were reared from the same host (*Plutella xylostella*). Performance of *D. semiclausum* was closely linked to that of its host, whereas the correlation between survival of *D. fenestrale* and host performance was less clear. Plants in the Brassicaceae characteristically produce defence related glucosinolates (GS). Levels of GS in leaves of undamaged plants were significantly higher in plants from the wild population than from the domesticated populations. Moreover, total GS concentrations increased significantly in wild plants after herbivory, but not in domesticated or feral plants. The results of this study reveal that a cabbage cultivar and plants from a wild cabbage population exhibit significant differences in quality in terms of their effects on the growth and development of insect herbivores and their natural enemies. Although cultivated plants have proved to be model systems in agroecology, we argue that some caution should be applied to evolutionary explanations derived from studies on domesticated plants, unless some knowledge exists on the history of the system under investigation.

Key words: crucifer, *Diadegma fenestrale*, *Diadegma semiclausum*, direct defence, glucosinolates, induction, *Mamestra brassicae*, *Pieris rapae*, *Plutella xylostella*

Introduction

The occurrence of pests in agroecosystems has long promoted the study of insect-plant interactions in crop plants, such as cabbage, lima bean, maize, cotton, and tomato (Takabayashi et al. 1994; Turlings et al. 1995; De Moraes et al. 1998; Geervliet et al. 2000; Thaler et al. 2001). Studies with crop plants have generated a wealth of data, highlighting a number of important mechanisms that influence the structure and function of multitrophic interactions and communities (Root 1973; Sheehan 1986; Khan et al. 1997; Gols et al. 2005). However, critics have argued that the biology and ecology of crop plants is often dramatically different from wild populations, thus bringing into some question the evolutionary relevance of the conclusions generated from data that rely on crop plants (Benrey et al. 1998; van der Meijden and Klinkhamer 2000). For instance, plant breeding programs have been reported to disrupt the original plant defence strategies that were present in the wild progenitors of cultivated species (Evans 1993). Artificial selection of some crop plants, aimed at accentuating a specific plant trait or group of traits (e.g., the production of edible structures), has been shown to reduce the level of undesired constituents, such as defence compounds, while enhancing others (such as primary metabolites including proteins and sugars). Many of the undesired secondary plant compounds are known to have evolved and function as putative defences against herbivores, whereas the desired primary plant compounds act as nutrients and thus may actually enhance the performance of herbivores (Schoonhoven et al. 2005).

Levels of secondary plant compounds are dynamic and vary with such factors as season, soil conditions and leaf age (reviewed by Schoonhoven et al. 2005). Moreover, plants may increase their levels of defences in response to feeding damage (Karban and Baldwin 1997; Agrawal 1999a), which may reduce the costs of defences by avoiding the allocation of resources to defence when the attacker is absent. Secondary plant compounds have also been shown to affect negatively the development of higher trophic levels that attack these herbivores such as predators, parasitoids and even hyperparasitoids (Barbosa et al. 1986; Barbosa et al. 1991; Francis et al. 2001b; Harvey et al. 2003, 2005, 2007a; Ode et al. 2004). Consequently, changes in plant chemistry, mediated by artificial selection, may influence the behaviour and development of consumers over several trophic levels, and this may ultimately lead to broader effects on the communities associated with these plants (Harvey et al. 2003; Ode 2006). Most importantly, in wild plants, defence mechanisms have not been constrained by the “directional selection” that characterises many crop plants. Therefore, to understand the evolution of plant defences against insect herbivores, multitrophic interactions should also be studied in

wild conspecifics of the cultivated plant species, where plant defences are the likely result of a range of biotic and abiotic selection pressures.

One appropriate plant family for studying the effects of artificial *versus* natural selection on multitrophic interactions is the Brassicaceae, which contains such important crops as cabbages and various types of mustard (Gómez-Campo and Prakash 1999). Of all plants that have been domesticated, few have been manipulated to produce so many different cultivars as *Brassica oleracea* L. (e.g., cabbage, broccoli, cauliflower and Brussels sprout Gómez-Campo and Prakash 1999). Wild types of *B. oleracea* grow naturally along rocky coastlines of Britain and France (Mitchell and Richards 1979). It has been speculated that the wild populations in the UK are derived from plants that were cultivated by the Greeks and Romans in the Mediterranean region between 1,000 and 2,000 BC (Mitchell 1976). These early cultivated forms were introduced to Britain, but have been naturalised for centuries (Mitchell 1976). However, more recent evidence points also at an Atlantic origin of domestication (Song et al. 1990).

Plants in the Brassicaceae characteristically produce secondary metabolites called glucosinolates (hereafter GS) (Fahey et al. 2001). After tissue damage, myrosinases catalyse the hydrolysis of GS into (iso)thiocyanates and nitriles (Fahey et al. 2001; Mithen 2001b), which play an important role in defence against insect herbivores (Rask et al. 2000). Generalist herbivores produce enzymes that can detoxify a wide range of substrates (Krieger et al. 1971), whereas specialists have evolved enzyme systems that can detoxify specific plant compounds that are associated with herbivore diet (e.g., Johnson 1999; Ratzka et al. 2002). Thus, generalist herbivores are usually more sensitive to high levels of specific allelochemicals compared to specialists (see e.g., Blau et al. 1978; Giamoustaris and Mithen 1995).

Specialist herbivores feeding on brassicaceous plants are adapted to feed on plants containing GS by detoxifying, excreting, or even sequestering these harmful metabolites (Müller et al. 2001; Ratzka et al. 2002; Wittstock et al. 2004). Moreover some insects may use these compounds as indicators of food plant suitability (Nayar and Thorsteinson 1963; Renwick and Lopez 1999). Not all GS are equally effective as stimulants and high levels of GS can reduce the performance of herbivores that are specialised on brassicaceous species (Stowe 1998; Li et al. 2000; Traw and Dawson 2002; Agrawal and Kurashige 2003). In addition, GS concentrations can increase in response to herbivore feeding damage (Bodnaryk 1992; Agrawal 1999b) and negatively effect subsequent herbivory by both generalists and specialists (Agrawal 1999b; Bartlett et al. 1999; Traw and Dawson 2002).

This study compares the development of several species of herbivores and endoparasitoids when reared on three *Brassica* populations that differ in their degree of domestication. Insects were reared on a cultivated and a wild population of *B. oleracea*, and a recently escaped (feral) *Brassica* species. Levels of GS were measured as indicators of direct plant defence. Initially, the development of two specialists on Brassicaceae, *Plutella xylostella* L. (Lepidoptera: Plutellidae) and *Pieris rapae* L. (Lepidoptera: Pieridae), and a generalist herbivore, *Mamestra brassicae* L. (Lepidoptera: Noctuidae), were examined when reared on the three populations. Finally, the development of a specialist and generalist parasitoid reared on the same host, *P. xylostella*, were compared. Separate cohorts of *P. xylostella* were parasitised by two species of endoparasitoids, *Diadegma semiclausum* Hellén (Hymenoptera: Ichneumonidae) and a related species, *D. fenestrale* Holmgren (Hymenoptera: Ichneumonidae). These two parasitoid species differ in host specialisation, with *D. semiclausum* restricted to *P. xylostella* and *D. fenestrale* attacking several other hosts that feed on non-brassicaceous species (Legaspi 1986; Azidah et al. 2000).

The following hypothesis was tested: specialist herbivores and parasitoids are less affected than generalists by differences in host plant chemistry between various *Brassica* populations that differ in their degree of domestication. It is proposed that changes in plant biology via domestication have significant effects on community level interactions and processes.

Methods and materials

Plants

The *B. oleracea* variety *gemmifera* (Brussels sprouts) cv. Cyrus was used. Compared with other vegetable crops of *B. oleracea*, Brussels sprouts cultivars contain relatively high levels of GS (Kushad et al. 1999; Rosa 1999), but considerably lower levels than the wild *B. oleracea* populations in Dorset, Great Britain (Moyes et al. 2000; Gols et al. 2008c). Seeds from several plants (>10) were collected from a wild population of *B. oleracea* growing on chalk cliffs along the south coast of Great Britain, near Swanage, Dorset ("Old Harry", 50°38'N, 1°55'E). This population contains intermediate levels of GS compared to other Dorset wild populations (Moyes et al. 2000). A feral *Brassica* population, which was found in a roadside hollow about 15 km east of Wageningen (51°57'N, 5°48'E, The Netherlands), was also included.

In addition to comparing the development of different herbivores, we also compared the development of a specialist and generalist parasitoid reared on the same host (see section on *Plutella xylostella* and *Diadegma* species). To discriminate

between food-plant quality mediated through the host and host quality itself, a second closely related wild brassicaceous plant species, black mustard, *Brassica nigra* L., was included in one of the experiments. Seeds of *B. nigra* were collected from a natural population growing in a small patch along the River Rhine, near Wageningen, The Netherlands (51°56'N, 5°37'E).

Seeds from the different populations were germinated in the first week of March 2005. Seedlings were transferred to 1.1 l pots filled with potting soil ("Lentse potgrond" no. 4, Lent, The Netherlands). Plants were grown in a greenhouse at 20–30 °C, 40–80% r.h., with a photoperiod of at least 16 h. If the light dropped below 500 $\mu\text{mol photons/m}^2/\text{s}$ during the 16-h photoperiod, supplementary illumination was supplied by high-pressure mercury lamps. Plants were watered daily. After the plants were 4 weeks old, they were fertilised once a week with Kristalon Blauw (N-P-K) 19-6-20-3 micro (2.5 mg/l), which was applied to the soil. *B. oleracea* plants were 7 weeks old when they were used in experiments and attained similar amounts of biomass (25–30 g per plant). Plants from all three populations were in the vegetative state and developed new leaves during the experiments. Fertilisation and watering continued during the experiments. *Brassica nigra* plants were 5 weeks old and were not fertilised, because the soil still contained enough nutrients for optimal growth. *B. nigra* matures much faster than *B. oleracea*.

Insects

All insects originated from cabbage fields in the vicinity of Wageningen. Cultures of all the herbivores have been maintained in the laboratory on Brussels sprouts cv. Cyrus for many years in climate rooms at 22 ± 2 °C, 40–80% r.h., with a light regime of 16: 8 L/D. The two parasitoid species were collected in the summer of 2004 and were thereafter reared on plants heavily infested with *P. xylostella* larvae for several generations. After pupation on the walls of the rearing cage, parasitoid cocoons were carefully removed and transferred to a clean cage. Emerged adult wasps were provided with water and honey *ad libitum*. For parasitism, we used females that were 5–10 days old after adult emergence.

Glucosinolate analyses

As an indicator of direct defence, GS concentrations in leaf tissues of *B. oleracea* were measured. Leaf samples were taken during the performance experiments (see below) from three treatment groups: plants that were undamaged, plants damaged by unparasitised *P. xylostella*, and plants damaged by larvae that had been parasitised by *D. semiclausum*. When leaf samples were taken, the damaged plant groups had been exposed to herbivore feeding for 7 days. Undamaged control plants were maintained in the same greenhouse, but were

physically separated from the plants with caterpillars. Larvae, feces, and pupae were removed from leaves. All fully developed leaves were harvested with the exception of the oldest leaves, which had turned yellow and did not contain feeding damage. Leaves were removed with a razor blade, pooled per plant, and stored at -80 °C. Samples were later freeze dried and pulverised with a mortar and pestle. Fifty milligram aliquots of freeze-dried material were weighed in 2-ml centrifuge tubes. GS were extracted and purified as described in van Dam et al. (2004) and were separated on a reverse phase C-18 column (Alltima C-18, 3 µm, 150 x 4.6 mm, Alltech, Deerfield, IL, USA) on HPLC (Dionex, Sunnyvale, CA, USA) with an acetonitrile water gradient. Detection was performed with a Dionex PDA-100 Photodiode array detector set to scan from 200 to 350 nm. For quantification, sinigrin (Sigma, St. Louis, MO, USA) was used as an external standard. Peaks were integrated at 229 nm for which standard response factors have been defined (EC 1990). The different GS were identified based on their retention times and UV spectra were compared to those of pure compounds (sinigrin, Sigma, St. Louis, MO, USA; glucotropaeolin and glucobrassicin were kindly provided by M. Reichelt, Max Planck Institute for Chemical Ecology, Jena, Germany), or compared to a certified oil seed reference (EC Community Bureau of Reference BCR-367R, Fluka, Buchs, Switzerland).

Insect performance

To investigate the effect of domestication on plant direct defences, the different herbivores and parasitoids (see below) were reared on the three plant populations. For all insects, egg-to-adult development time, adult dry mass, and survival (to adult) were determined when reared on the different populations. Adult dry mass was obtained by weighing adults on a Cahn C-33 microbalance (Cahn Instruments, Cerritos, CA, USA) that had been dried to constant weight at 80 °C (3 days). Plants with insects were maintained in a greenhouse under the same conditions as described in the *Plant* section. Plants of the same population that received the same insect treatment were placed together, and caterpillars were allowed to develop and move around freely on plants until they reached the final instar. Different herbivore treatments were randomly positioned in a greenhouse, but were all placed in a similar position relative to the light sources to minimise microclimatic differences among plant populations and treatments.

***Plutella xylostella* and *Diadegma* species**

To obtain eggs of *P. xylostella*, more than 150 adult moths were released with a 50:50 sex ratio in a plastic cage (37 x 40 x 30 cm). Folded strips of Parafilm served as substrate for females to lay eggs on. Females were allowed to oviposit on the Parafilm

overnight. Subsequently, the strips with eggs were incubated for 4 days at 22 °C until the eggs hatched. Pieces of Parafilm with neonate larvae were placed on top of individual plants of each of the three plant populations. Larvae were allowed to feed on these plants until they reached the third larval instar (L3).

For each plant population, one cohort of 60 larvae was transferred to new plants and served as an unparasitised control. A second cohort of 130 larvae was parasitised by *D. semiclausum*, and a third cohort of 180 larvae was parasitised by *D. fenestrale*. For parasitism, individual female wasps of both species were presented with a L3 *P. xylostella* host. A host was considered as parasitised when the female wasp was observed to insert into and remove her ovipositor from the larva. Individual female wasps were allowed to oviposit in up to 10 separate hosts. After this, they were removed. Parasitised larvae were transferred to new plants of the same population on which the larvae had fed previously. Five plants were used for the unparasitised controls and nine plants for each of the parasitoid treatments. The number of plants provided ample food for all larvae to complete their development.

When caterpillars molted into L4, strips of corrugated cardboard were placed on top of the plants, as *P. xylostella* prefers to pupate in secluded areas. After pupation, the cocoons were collected and stored in labelled vials until adult emergence. When the moths or wasps emerged, the time of eclosion and sex were recorded. Individuals were killed by freezing at -20 °C and stored for dry mass determination. Vials with cocoons ready to emerge were checked every 2 h. Development time for *P. xylostella* was measured in full days, as the exact time of oviposition had not been recorded. In the case of the two *Diadegma* species, the median time point of the period needed to parasitise the hosts (3-4 h), was used as the time of oviposition.

To further investigate whether food plant quality or host quality was a more important factor in the development of *D. fenestrale*, the experiments described above with *P. xylostella* were repeated on a second (and closely related) wild brassicaceous species, *B. nigra*. A separate study (Gols et al. unpublished) has shown that *B. nigra* is a qualitatively superior plant for the development of *P. xylostella*, compared with *B. oleracea*. We reared 33 unparasitised larvae on three *B. nigra* plants and 180 larvae parasitised by *D. fenestrale* on 10 plants. We recorded egg-to-adult development time, adult biomass and survival as before. The plants provided ample food for all the larvae to complete their development.

Pieris rapae

Neonate larvae were obtained from the general culture and transferred to seven plants of each population with a distribution

of six larvae per plant. When larvae had developed into L5, they were transferred to plastic containers that contained some leaf material from the plants they had fed on previously. After pupation, pupae were collected and placed in new plastic containers lined with filter paper. At adult emergence, the time of eclosion and sex were recorded, and the individuals were killed by freezing, followed by dry mass determination (as above). Containers with pupae ready to emerge were checked every 2 h. Development time was measured in full days, as the exact time of oviposition had not been recorded.

Mamestra brassicae

Like *P. xylostella*, adult *M. brassicae* are primarily nocturnal. Females do not need plants as an oviposition substrate and readily lay batches of eggs onto the surface of paper. From the general culture, we obtained paper sheets with *M. brassicae* eggs that were laid the previous night. These sheets were incubated at $22 \pm 2^\circ\text{C}$ (5 days) until the eggs hatched. Neonate larvae were transferred to 10 plants of each population, with a density of five larvae per plant. Once they had reached late L5, *M. brassicae* larvae were collected from the three populations, counted, and transferred to plastic containers (15x12x6 cm) that contained 2 cm of potting soil mixed with vermiculite (1:1) and some leaf material from the plant on which they had been feeding previously. After the larvae had pupated, they were collected and placed in new plastic containers filled with a layer of vermiculite. At moth emergence, the date of eclosion was recorded, and the individuals were killed by freezing, followed by dry mass determination. Containers with pupae ready to emerge were checked every 2 h. Development was measured in full days as the exact time of oviposition had not been recorded.

Statistical analysis

Data on adult dry mass and development time were analyzed by using ANOVA with plant population and sex and their interaction as factors. All larvae within one plant population were considered as independent samples. The Tukey-Kramer method was used for multiple comparisons of the means. For each insect species, a G test for heterogeneity was performed on survival rates on the three plant populations with H_0 : survival on each of the three plant populations is equal.

Concentrations of individual GS compounds were $\log(x+1)$ transformed to meet assumptions of normality. To examine differences in GS content, a Mixed Model was used with plant population and plant treatment (intact, damaged by unparasitised *Plutella*, damaged by parasitised *Plutella*) as the fixed factors in the model. There was no random factor in the analysis, and the estimation of effects in the model was based on restricted likelihood maximisation. When the main factors

or their interactions were significant, specific linear contrasts were applied to separate further factor levels. When necessary, correction for unbalanced sample sizes was carried out by using the Satterthwaite correction. Analysis was carried with SAS 8.02 (1999-2001 ©SAS Institute, Inc).

Results

Glucosinolate analyses

GS analyses of leaf tissues revealed considerable quantitative and some qualitative variation among the different *Brassica* populations (Fig. 1). Three pentyl-derived (C5) GS, glucoallyssin, gluconapoleiferin, and glucobrassicinapin, were only detected in the feral population, whereas the other 10 compounds were present in all three populations (Fig. 1). Total GS concentrations in undamaged plants were 3.2 and 1.4 times higher in plants of the wild population than in the cultivated and feral population, respectively (Fig. 1). Furthermore, concentrations of all

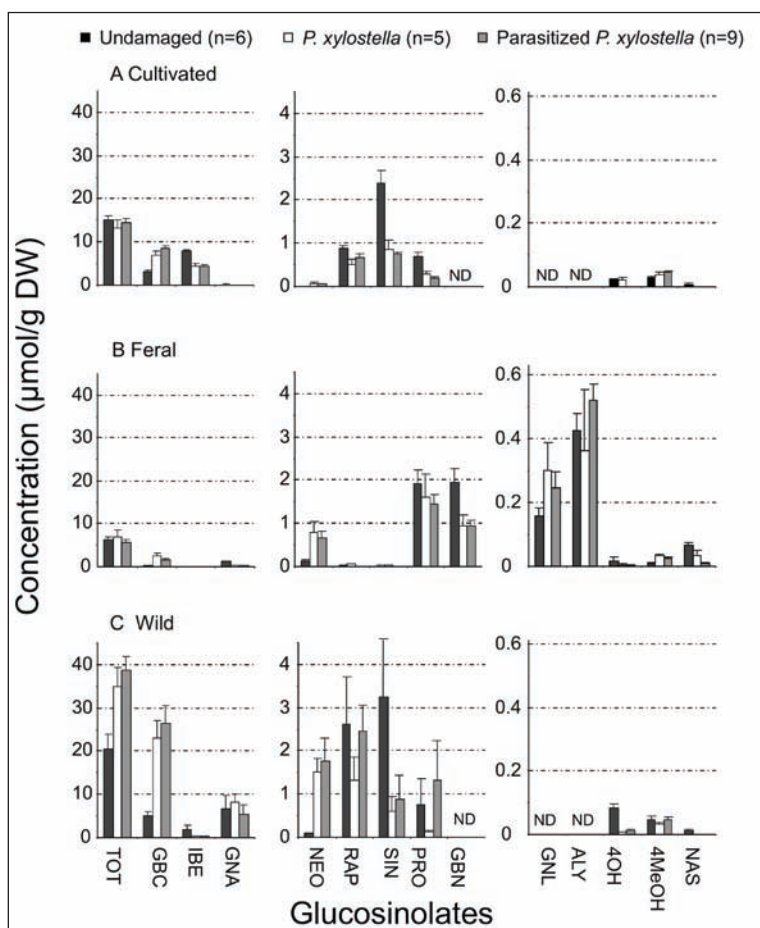
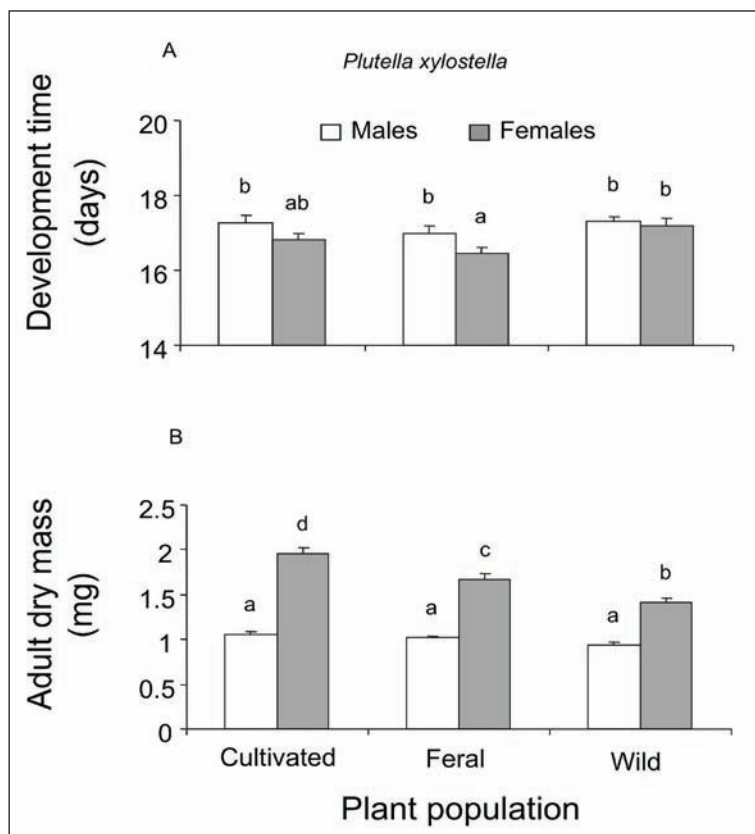


Figure 1: Glucosinolate (GS) concentrations (mean ± SE) in leaf tissues of a cultivated (A), feral (B), and wild (C) *Brassica* population. Concentrations were measured in leaf tissue from plants that were undamaged (black bars), damaged by unparasitised *P. xylostella* larvae (white bars) and damaged by parasitised (*D. semiclausum*) larvae (grey bars). GS abbreviations and scientific names: TOT, total GS concentration; GBC, glucobrassicin (= indol-3-ylmethyl GS); IBE, glucoiberin (= 3-methylsulfinyl propyl GS); GNA, gluconapin (= 3-butenyl GS); NEO, neoglucobrassicin (= 1-methoxyindol-3-ylmethyl GS); RAP, glucoraphanin (= 4-methylsulfinyl butyl GS); SIN, sinigrin (= 2-propenyl GS); PRO, progoitrin (= 2(R)-2-hydroxy-3-butenyl GS); GBN, glucobrassicinapin (= 4-pentenyl GS); GNL, gluconapoleiferin (= 2-hydroxy-4-pentenyl GS); ALY, glucoallyssin (= 5-methylsulfinyl pentyl GS); 4OH, 4-hydroxyglucobrassicin (= 4-hydroxyindol-3-ylmethyl GS); 4MeOH, 4-methoxyglucobrassicin (= 4-methoxyindol-3-ylmethyl GS) and NAS, gluconasturtiin (= 2-phenylethyl GS). ND: not detectable

Figure 2: Egg-to-adult development time (A) and adult dry mass (B) of *P. xylostella* males (white bars) and females (grey bars) reared on either a cultivated, a feral, or wild *Brassica* population. Bars (mean + SE) with different letters are significantly different from each other (Tukey multiple comparisons, $\alpha = 0.05$). Numbers of individuals (n) were on the cultivar, escape, and wild population, respectively: males, 21, 22, and 31; females 23, 22, 18.



Differences between the cultivated and feral populations on the one side, and the wild population on the other side, became even more pronounced after the plants were induced by larval *P. xylostella* feeding (Fig. 1). In the cultivated and feral plants, total levels of GS remained at similar levels before and after induction by *P. xylostella* feeding (cultivar: $t_{50} = 0.82$, $P = 0.42$; feral population: $t_{50} = 0.39$, $P = 0.70$), whereas in the wild population, concentrations were 1.5-2 times higher after herbivore feeding ($t_{50} = 3.11$, $P < 0.001$). Levels of individual GS changed differentially in response to herbivore feeding. The indole GS, glucobrassicin, was significantly induced by *P. xylostella* feeding in all the plant populations ($t_{50} = 6.90$, $P < 0.001$). Moreover, glucobrassicin accounted for almost 70% of the GS composition in the wild population after induction, and for only 53% and 35% in the cultivated and feral population, respectively. In contrast, the relative amount of glucobrassicin in undamaged plants was only 21%, 5% and 25% in the cultivated, feral, and wild population, respectively. A second indole GS, neoglucobrassicin, was also

induced in response to herbivory in the feral ($t_{50} = 2.55$, $P = 0.01$) and the wild population ($t_{50} = 4.76$, $P < 0.001$), but not in the cultivated population ($t_{50} = 0.37$, $P = 0.71$) in which levels of this compound were very low.

Not all GS concentrations increased after herbivory. Sinigrin was reduced after *P. xylostella* larval feeding in the cultivated ($t_{50} = 2.4$, $P = 0.02$) and the wild population ($t_{50} = 3.67$, $P < 0.001$), but not in the feral population ($t_{50} = 0.07$, $P = 0.95$). Similarly, levels of glucoiberin decreased in response to *P. xylostella* feeding in the cultivar ($t_{50} = 2.79$, $P = 0.007$) and the wild population ($t_{50} = 4.22$, $P < 0.001$), but not in the feral population ($t_{50} = 0.02$, $P = 0.99$). In the feral population, both sinigrin and glucoiberin were present in much lower concentrations than in the other two populations (Fig. 1). In plants damaged by parasitised and unparasitised larvae of *P. xylostella*, concentrations of individual GS were not significantly different (statistics not shown, but all significance levels were lower than 0.05).

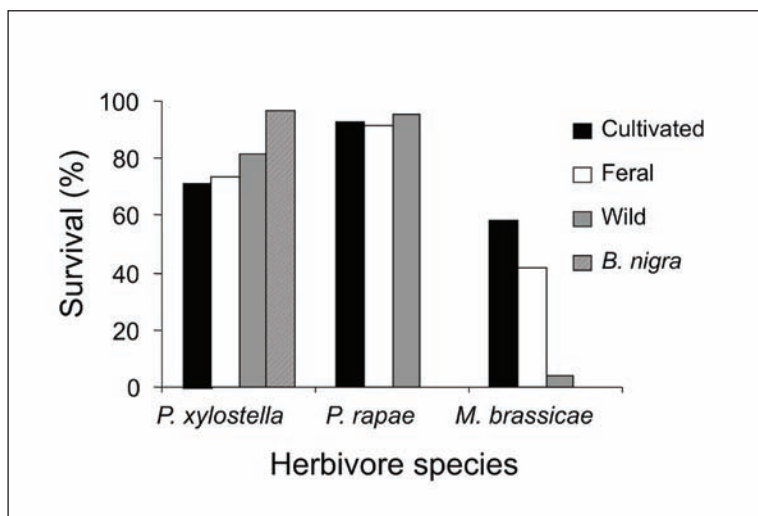
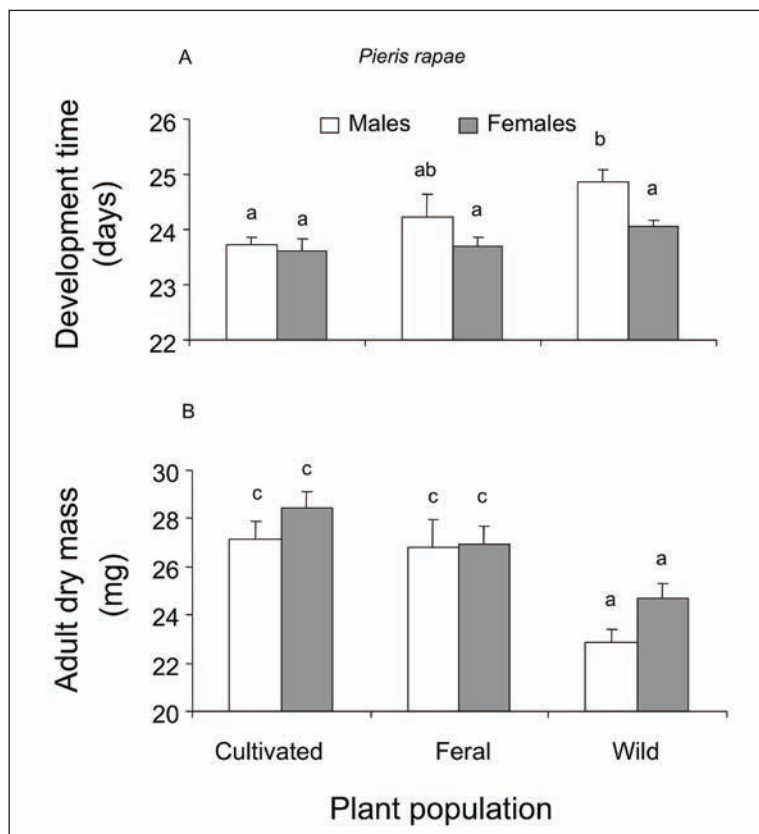


Figure 3: Larval to adult survival of two specialist herbivores, *P. xylostella* and *P. rapae*, and one generalist, *M. brassicae*, when reared on either a cultivated, feral, or wild *Brassica* population. *P. xylostella* was also reared on a wild population of *B. nigra*.

Insect performance: herbivores

In *P. xylostella*, plant population and sex had an effect on egg-to-adult development time (plant population: $F_{2,131} = 5.95$, $P = 0.003$; sex: $F_{1,131} = 5.52$, $P = 0.02$; Fig. 2A). Female *P. xylostella* developed faster than males; the fastest development time was observed for females reared on the cultivated and the feral population. For adult biomass, the interaction between plant population and sex was significant ($F_{2,132} = 11.1$, $P < 0.001$). Plant population had a strong effect on female, but not on male biomass (Fig. 2B). The heaviest females were recovered from the cultivar, and the lightest from the wild population. In contrast, males were significantly lighter than females ($F_{1,132} =$

Figure 4: Egg-to-adult development time (A) and adult dry mass (B) of *P. rapae* males (white bars) and females (grey bars) reared on either a cultivated, feral, or wild *Brassica* population. Bars (mean + SE) with different letters are significantly different from each other (Tukey multiple comparisons, $\alpha = 0.05$). Numbers of individuals (n) were on the cultivated, feral, and wild population, respectively: males, 22, 10, and 19; females 16, 20, 20.



317, $P < 0.001$) and obtained similar biomasses on all three plant populations (Fig. 2B). For *P. xylostella*, plant population did not affect larval survival to the adult stage ($\chi^2 = 0.96$, d.f. = 2, $P = 0.62$, Fig. 3).

Development time also varied with the population on which the *P. rapae* larvae had been reared ($F_{2,105} = 7.8$, $P < 0.001$; Fig. 4A). Egg-to-adult development time of males was shortest on the cultivar, longer on the feral and longest on the wild population (Fig. 4A). Males took longer to complete their development than females ($F_{1,105} = 7.5$, $P = 0.007$). Plant population also had a significant effect on adult biomass ($F_{2,101} = 17.5$, $P < 0.001$; Fig. 4B). Whereas adult biomass in *P. rapae* did not differ between the cultivated and the feral line, biomass of butterflies reared on the wild population was lower (Fig. 4B). On average, females were marginally heavier than males ($F_{1,101} = 3.2$, $P = 0.08$). Irrespective of plant population, more than 92% of all *P. rapae* larvae successfully developed into adults and survival rates were not significantly different ($\chi^2 = 0.22$, d.f. = 2, $P = 0.90$; Fig. 3).

In the case of the generalist herbivore, *M. brassicae*, the effect of host-plant population on adult biomass was much more pronounced compared to the two specialists. It is difficult to determine the sex of adult moths, therefore, the data were

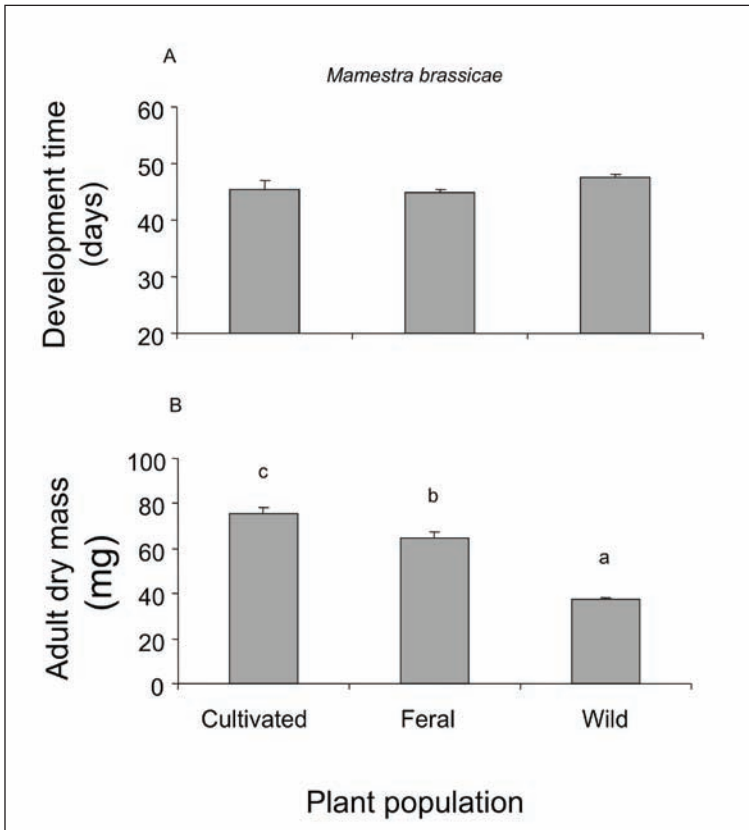


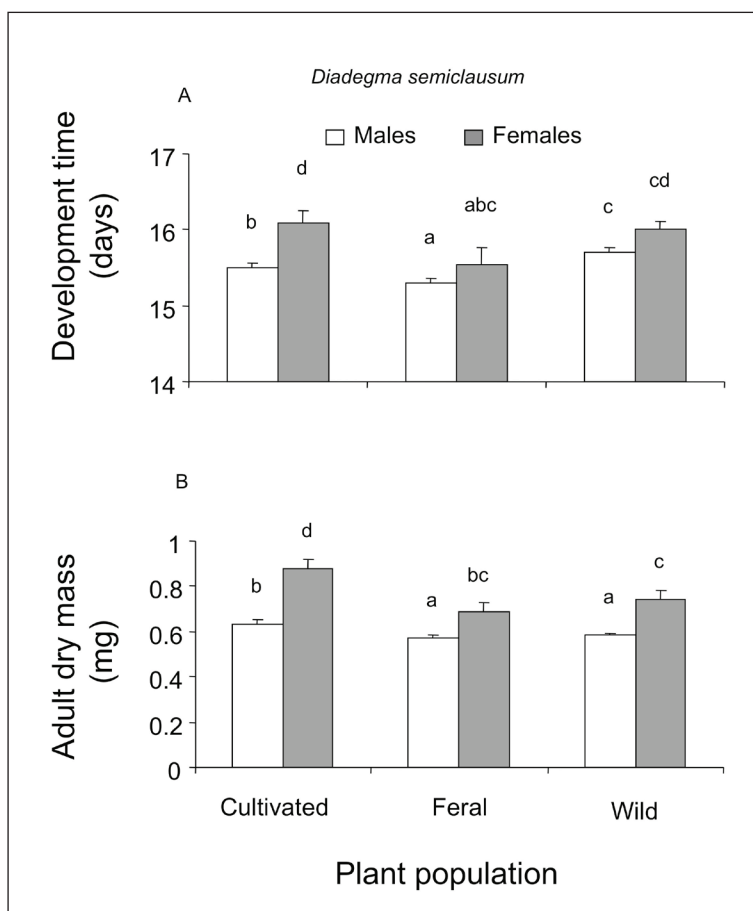
Figure 5: Egg-to-adult development time (A) and adult dry mass (B) of *M. brassicae* reared on either a cultivated, feral, or wild *Brassica* population. Bars (mean + SE) with different letters are significantly different from each other (Tukey multiple comparisons, $\alpha = 0.05$). Numbers of individuals (n) were on the cultivated, feral, and wild population, respectively: 21, 29, and 2.

pooled. Adult mass on the cultivated line was twice as high, compared to the wild population, and was also significantly higher on the feral population ($F_{2,49} = 8.28$, $P < 0.001$; Fig. 5B). Plant population did not affect development time ($F_{2,49} = 0.33$, $P = 0.72$; Fig. 5A). Unlike the two specialists, survival of *M. brassicae* was affected by plant population ($\chi^2 = 20.4$, d.f. = 2, $P < 0.001$; Fig. 3). The percentage of *M. brassicae* larvae that developed successfully into adults was highest on the cultivated population (58%), slightly lower on the feral population (42%), and smallest (4%) on the wild population.

Insect performance: parasitoids

In the specialist parasitoid, *D. semiclausum*, plant population had an effect on egg-to-adult development time ($F_{2,275} = 6.49$, $P = 0.002$; Fig. 6A). The parasitoid developed fastest on the feral population and developed more slowly on the cultivated and wild populations of *B. oleracea* (Fig. 6A). Egg-to-adult development time was longer in females than in males ($F_{1,275} = 13.0$, $P < 0.001$). Furthermore, plant population had an effect on adult biomass in *D. semiclausum*, ($F_{2,277} = 11.3$, $P < 0.001$; Fig. 6B). The heaviest *D. semiclausum* wasps emerged from

Figure 6: Egg-to-adult development time (A) and adult dry mass (B) of *D. semiclausum* males (white bars) and females (grey bars) reared on *P. xylostella* feeding on either a cultivated, feral, or wild *Brassica* population. Bars (mean $\pm$ SE) with different letters are significantly different from each other (Tukey multiple comparisons, $\alpha = 0.05$). Numbers of individuals (n) were on the cultivated, feral, and wild population, respectively 87, 69, and 81 for males and 11, 12, 10 for females.



hosts that were reared on the cultivated population, whereas the lightest emerged from host reared on the wild and feral populations. Further, *D. semiclausum* females were heavier than males ($F_{1,277} = 57.4$, $P < 0.001$). Between 67 and 80% *D. semiclausum* successfully completed development to eclosion on the three *Brassica* populations (Fig. 7). Survival rates were not significantly different on the three plant populations ($\chi^2 = 3.77$, d.f. = 2, $P = 0.15$).

As with *D. semiclausum*, egg-to-adult development time in *D. fenestrata* varied with plant population ($F_{2,69} = 5.27$, $P = 0.007$; Fig. 8A). However, dry mass in male *D. fenestrata* wasps did not vary significantly with the population on which the hosts had been reared ($F_{2,69} = 0.25$, $P = 0.78$; Fig. 8B). As only thirteen *D. fenestrata* females in total successfully developed, data on females were excluded from the analysis and are not presented in the figures. The most dramatic effect of plant population was on the survival of *D. fenestrata* (Fig. 7), which was significantly different for the three plant populations ($\chi^2 = 12.2$, d.f. = 2, $P = 0.002$). Only 9% of the parasitised hosts reared on the wild population successfully produced *D. fenestrata* wasps. By contrast, 33% survived on the

cultivar and 16% on the feral population. Moreover, the lowest number of surviving *D. fenestrale* parasitoids, eight out of 172 (or 4.6%), was obtained on *B. nigra*. Male wasps ($n = 5$) developing in hosts reared on *B. nigra* plants were lighter (0.432 ± 0.033 mg, mean $\pm$ SE) and developed slower (17.8 ± 0.37 days) than males reared from hosts on *B. oleracea* (see Fig. 6). In contrast, healthy *P. xylostella* moths performed better on *B. nigra* than on *B. oleracea*, in terms of survival (97%, Fig. 3) and adult mass, 1.985 ± 0.068 mg ($n = 16$) and 1.104 ± 0.068 mg ($n = 16$), for females and males, respectively (see also Fig. 2B). However, development time, 17.8 ± 0.26 days for both female and male moths, was slightly longer (see also Fig. 2A).

Discussion

Through artificial selection via domestication, levels of primary and secondary compounds in domesticated plants are often altered compared with their progenitors. The results of this study revealed that GS concentrations in leaf tissue varied significantly among the three different plant populations. Much higher concentrations of GS were recorded in wild *B. oleracea* than in the cultivated and feral population. GS levels in the wild population studied in this paper were similar to concentrations reported earlier in other wild populations of *B. oleracea* (Mithen et al. 1995; Moyes et al. 2000). The most striking differences in GS concentrations were observed after induction by herbivore feeding, especially in the wild population. In wild plants, total GS concentrations were 1.7 times higher in induced plants than in uninduced conspecific plants and were 2.7 and 4.9 times higher after herbivory than in induced plants of the cultivated and feral population, respectively. By contrast, the total GS concentrations in induced plants remained at the same level as in undamaged plants in the cultivated and feral populations, although differences were found for levels of individual compounds.

The feral population differed from the other two populations with respect to pentyl-derived GS, which were absent in the cultivated and wild population. These C5 GS usually are not found in *B. oleracea* crops (Rosa 1999), indicating that this population may have crossed with a closely related species such as *B. napus* or *B. rapa*, both of which contain pentyl GS (Giamoustaris

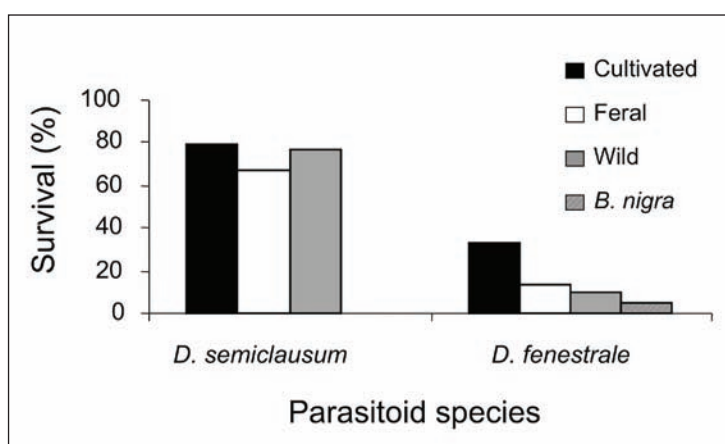
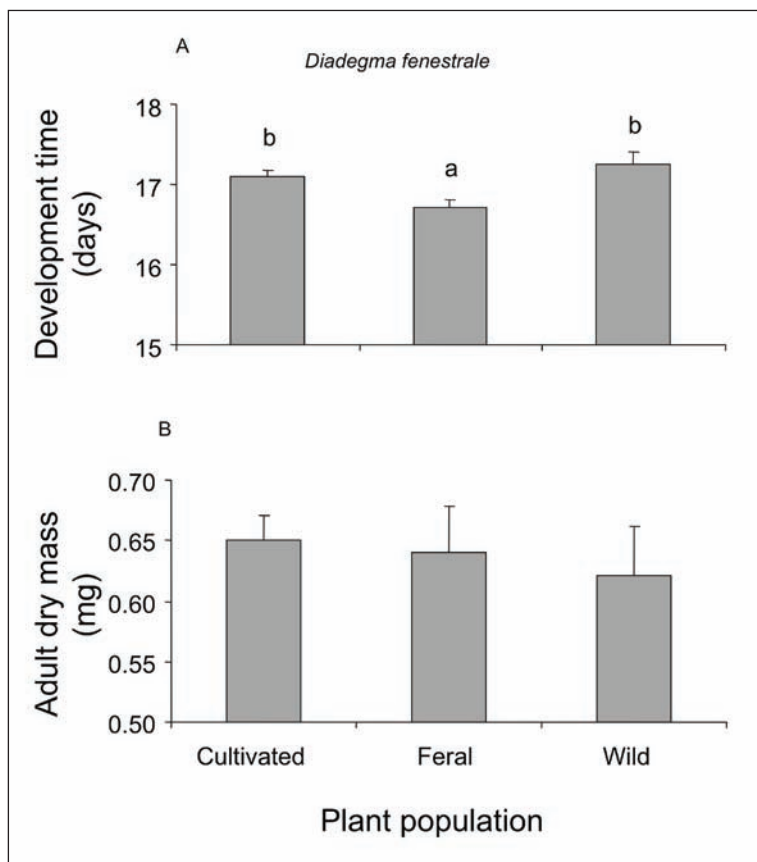


Figure 7: Egg to adult survival of a specialist parasitoid, *Diadegma semiclausum*, and a generalist parasitoid, *D. fenestrale*, when reared from the host, *P. xylostella*, on either a cultivated, feral, or wild *Brassica* population. *D. fenestrale* was also reared from *P. xylostella* on a wild population of *B. nigra*.

Figure 8: Egg-to-adult development time (A) and adult dry mass (B) of *D. fenestrale* males reared on *P. xylostella* feeding on either a cultivated, feral, or wild *Brassica* population. Bars (mean + SE) with different letters are significantly different from each other (Tukey multiple comparisons, $\alpha = 0.05$). Numbers of individuals (n) were on the cultivar, escape, and wild population respectively: 44, 18, and 14.



and Mithen 1995). Moreover, both *B. napus* and *B. rapa* are cultivated and grow naturally in the Netherlands. In all plant populations, concentrations of the indole GS, glucobrassicin, increased the most after herbivore feeding, but this compound was dominant only in the wild population, accounting for 70% of the GS content. Several studies have reported on herbivore-induced changes in GS concentrations in both cultivated and wild brassicaceous species. In line with our results, previous studies have shown that levels of indole GS increase in response to insect wounding (Bodnaryk 1992; Agrawal et al. 1999b; Bartlet et al. 1999; Gols et al. 2008c). We found that concentrations of some of the aliphatic GS were lower in plants that had been damaged by *P. xylostella* than in undamaged plants. Previous studies in which different herbivores were used, reported that levels of aliphatic GS were either unaffected (Bodnaryk 1992; Bartlet et al. 1999; Traw and Dawson 2002; Gols et al. 2008c) or even increased (Traw and Dawson 2002) in response to insect wounding. These results suggest differential induction of aliphatic GS by different herbivores (see also Traw and Dawson 2002).

Glucosinolates also play a major role in determining a

plant's nutritional quality, not only for humans and livestock but also for pathogens and insect herbivores (Chew 1988; Mithen 1992). This study shows that plants from a wild population of *B. oleracea* are much less suitable for the development of several herbivores than plants from a cultivated and a feral population. However, the severity of these effects differed between the specialists *P. rapae* and *P. xylostella* on one hand, and the generalist *M. brassicae* on the other. In *P. rapae*, emerging adult butterflies were smaller and took longer to complete development when reared on wild plants than on the other two populations. These effects were less pronounced in *P. xylostella*; however, where adult body mass was more negatively affected than development time. Importantly, in both of the specialist herbivores, survival was high, irrespective of the plant population on which the larvae had been reared. By contrast, adult body mass and survival in the generalist herbivore, *M. brassicae*, were significantly lower when reared on the wild *B. oleracea* strain. This reveals that costs in terms of reduced fitness are higher for generalist herbivores than for specialists when they feed on the more toxic wild plants. Similarly, Giamoustaris and Mithen (1995) found a negative relationship between GS content in oilseed rape (*B. napus*) and the amount of leaf damage by generalist herbivores, but a positive relationship for specialists. Levels of GS, especially indole GS, were higher in the wild population of *B. oleracea* than levels of GS found in *B. napus* by Giamoustaris and Mithen (1995). These results suggest that indole GS, especially neoglucobrassicin, which is present in very low concentrations in the cultivar, may play a role in reducing the performance of insect herbivores.

Specialist insect herbivores, in contrast with generalists, may use GS as indicators of host plant suitability. For example, GS serve as feeding stimulants for insect herbivores specialised on plants belonging to the Brassicaceae (Nayar and Thorsteinson 1963; David and Gardiner 1966; Renwick and Lopez 1999). However, not all GS are equally active as feeding stimulants (e.g., Nayar and Thorsteinson 1963), and high levels of GS can even be toxic for specialists (Agrawal and Kurashige 2003). As such, high levels of specific GS may be responsible for the reduced performance of the specialists *P. xylostella* and *P. rapae* when reared on the wild population. Furthermore, levels of the enzyme myrosinase, which catalyzes hydrolysis of GS into the more toxic (iso)thiocyanates and nitriles (Mithen 2001b; Rask et al. 2000), may also have differed among the populations. In addition, host plant quality is not determined only by the presence of allelochemicals. Nutrients, such as proteins and carbohydrates, as well as digestibility reducers, also play a role (Slansky 1993). It is possible that levels of limiting nutrients such as nitrogen and other defence-related compounds also vary across the three populations and thus amplify differences

in performance caused by GS (Slansky and Feeny 1977).

The performance of the two parasitoid species reared on *P. xylostella* also varied with the plant population on which the host had been reared. However, there were also significant differences in performance between the specialist parasitoid, *D. semiclausum*, and the congeneric generalist parasitoid, *D. fenestrale*. The development of *D. semiclausum* was directly affected by the development of the host. Adult body mass was reduced when developing on wild *B. oleracea* plants, whereas development time and survival were unaffected, revealing that *D. semiclausum* ontogeny is affected by quantitative changes in host quality as mediated through the diet of their host. Alternatively, the development of *D. fenestrale* was characterised less by direct differences in host quality than by indirect population-related variations in plant quality. Although *P. xylostella* survival was high (>80%) on all *B. oleracea* populations, as well as on *B. nigra* plants, mortality in *D. fenestrale* was much higher on the wild *Brassica* population.

As in most endoparasitoids, larvae of *D. fenestrale* primarily consume host haemolymph and fat body during early development. They only begin to indiscriminately attack other tissues later during development (the so-called “destructive feeding phase”). In this way, they do not kill the host until the last possible moment. Larvae of *P. xylostella* are known to utilise enzymes that convert GS into desulfo-GS in their gut, which are then excreted with their feces (Ratzka et al. 2002). Because of their polarity, GS presumably do not permeate the host-gut membrane but effectively remain in the gut before they are excreted. Consequently, the larvae of *D. fenestrale* probably ingest little, if any, GS when feeding on haemolymph. However, during the destructive feeding phase, the parasitoid larvae undoubtedly consume the host gut and its contents, and this is when the toxic effects of plant allelochemicals on non-adapted parasitoids may be realised. In hosts parasitised by *D. fenestrale*, mortality mainly occurred just before the parasitoids would have been expected to pupate (personal observation) supporting this argument. Furthermore, the gut of endoparasitoid larvae is not externally connected until after emergence from the host. The excretion of wastes into internal host tissues would facilitate bacterial infection and precocious death of both the host and the developing parasitoid (Harvey et al. 2003). Thus, low concentrations of allelochemicals that are ingested by parasitoid larvae are stored and presumably accumulate in their tissues. This may account for the high mortality recorded here with *D. fenestrale* that developed in *P. xylostella* caterpillars reared on mustard and wild cabbage plants, which contain high levels of GS.

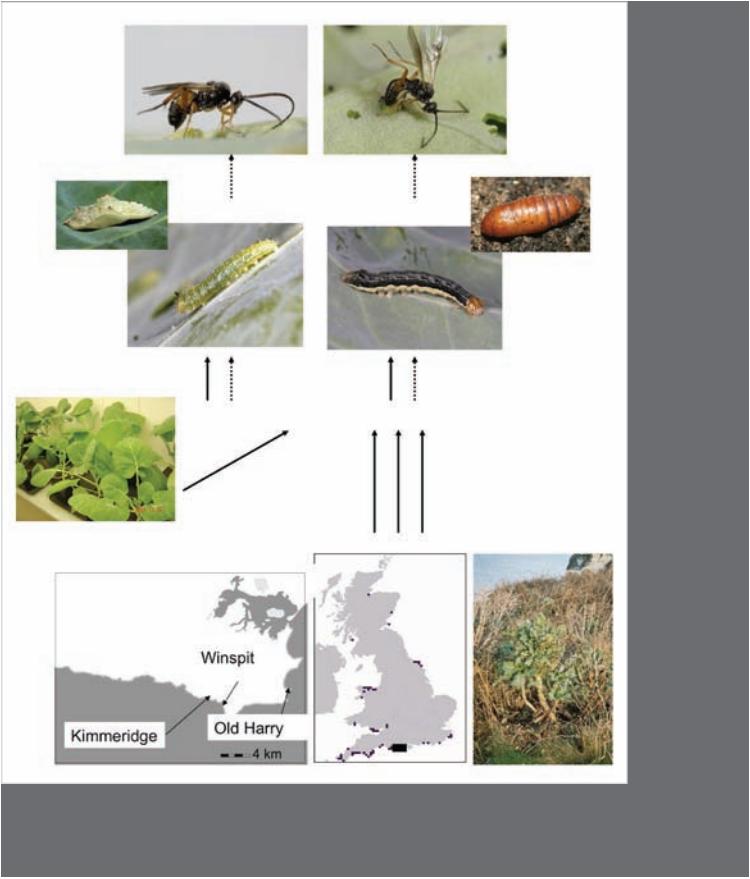
All insects used in this study have been reared on the Cyrus cultivar for many generations and may have adapted to

this plant population. However, *P. xylostella* developed more successfully on *B. nigra* plants than on the cultivar, with moths enjoying higher survival and larger body mass. Furthermore, the performance of *Mamestra brassicae* and *Pieris rapae* was almost similar on the feral and the cultivated population. We cannot exclude that the observed differences are the result of rearing history rather than true plant effects, but the fact that the insects have no history with the feral *Brassica* population and developed with equal success as on the cultivar suggests that plant quality is affected by domestication. Moreover, the development of the insects was more strongly negatively affected on the wild “Old Harry” population than on the feral population.

In summary, this study has shown that plant quality in terms of development of herbivores and their natural enemies differs significantly between wild and cultivated populations of *B. oleracea*. The identity of the food plant and the degree of specialisation exhibited by the herbivores and their parasitoids influenced the degree to which plant population affected performance. Most importantly, these results demonstrate that artificial selection in which certain plant traits are accentuated at the expense of others can alter a significant part of a plant's evolved physiology. This may in turn have large impacts on insect communities that are associated with these plants. In wild plants, defence mechanisms have evolved under natural selection pressures from herbivores and pathogens and by the effects of natural enemies on herbivore populations. The reduction of the levels of direct defences in cultivated plants could partly explain why these plants have often become more susceptible to attack from a wide range of herbivores and pathogens. To better understand the relative contribution of insect herbivory as a selective agent on the evolution of plant defences, these traits should be studied in wild populations in which defence mechanisms have not been constrained by the “directional selection” that characterises many species of crop plants. Future studies should examine insect communities associated with plant populations, including cultivars that differ in resistance against insect herbivores in plots in which the structural heterogeneity is also manipulated. This will facilitate a better understanding of the role that artificial selection has played in shaping the structure of communities associated with cropping systems.

Acknowledgements

The authors would like to thank André Gidding and Leo Koopman for rearing the insects and Unifarm for rearing the plants. Ciska Raaijmakers from the Netherlands Institute of Ecology is acknowledged for her assistance in the GS analyses.



5

Genetic variation in defence chemistry in wild cabbages affects herbivores and their endoparasitoids

by R. Gols, R. Wagenaar, T. Bukovinszky, N.M. van Dam,
M. Dicke, J.M. Bullock & J.A. Harvey

To be published in *Ecology* (in press)

Populations of wild *Brassica oleracea* L. grow naturally along the Atlantic coastlines of the United Kingdom and France. Over a very small spatial scale, i.e. less than 15 km, these populations differ in the expression of the defensive compounds, glucosinolates (GS). Thus far, very few studies have examined interactions between genetically distinct populations of a wild plant species and associated consumers in a multitrophic framework. Here, we compared the development of a specialist (*Pieris rapae*) and a generalist (*Mamestra brassicae*) insect herbivore and their endoparasitoids (*Cotesia rubecula* and *Microplitis mediator*, respectively) on three wild populations and one cultivar of *B. oleracea* under controlled greenhouse conditions. Herbivore performance was differentially affected by the plant population on which they were reared. Plant population influenced only development time and pupal mass in *P. rapae*, whereas plant population had also a dramatic effect on survival of *M. brassicae*. Prolonged development time in *P. rapae* corresponded with high levels of the indole GS, neoglucobrassicin, whereas reduced survival in *M. brassicae* coincided with high levels of the aliphatic GS, gluconapin and sinigrin. The difference between the two species can be explained by the fact that the specialist *P. rapae* is adapted to feed on plants containing GS and has evolved an effective detoxification system against aliphatic GS. The different *B. oleracea* populations also affected development of the endoparasitoids. Differences in food-plant quality for the hosts were reflected in adult size in *C. rubecula* and survival in *Mi. mediator*, and further showed that parasitoid performance is also affected by herbivore diet.

Keywords: bottom-up effects, *Brassica oleracea*, co-evolution, *Cotesia rubecula*, glucosinolates, *Mamestra brassicae*, *Microplitis mediator*, plant defence, *Pieris rapae*, top-down effects

Introduction

Plant defence strategies against insect herbivores and pathogens often involve the production of allelochemicals (secondary plant metabolites), which are usually phylogenetically conserved in specific plant families or genera, e.g., coumarins in Umbelliferae, tropane alkaloids in Solanaceae and glucosinolates in the Brassicaceae (Rosenthal and Berenbaum 1992; Rask et al. 2000; Schoonhoven et al. 2005). A vast amount of literature has demonstrated that many secondary plant compounds are active against insect herbivores. These compounds can act as feeding deterrents or significantly alter the physiology and development of some herbivores, through reduced growth rates, smaller adult size and increased mortality (Zangerl and Berenbaum 1993; Li et al. 2000; van Dam et al. 2000). Conversely, many specialist herbivores are known to exhibit resistance to secondary plant compounds and may require them as feeding stimulants (Bowers 1983; Renwick 2002; Schoonhoven et al. 2005). Some resistant herbivores sequester plant toxins in their haemolymph or body tissues, thus providing them with some degree of protection from their natural enemy complex (Nishida 2002).

Insect herbivore populations are not only affected by plant characteristics (bottom-up effects), but also by the presence of natural enemies such as predators and parasitoids (top-down effects). The relative strength of bottom-up and top-down control of insect herbivores in natural ecosystems has been the subject of debate over many years (Hairston et al. 1960; Keeler et al. 2006; Gripenberg and Roslin 2007). Many studies examining the effects of plant defence on insect herbivores have either focused exclusively on interactions between plants and insect herbivores (direct defence) or between plants and natural enemies of insect herbivores (indirect defence). Secondary plant compounds have been shown to negatively affect the development of organisms in the third (Campbell and Duffey 1979; Barbosa et al. 1991; Roth et al. 1997) and even the fourth (Orr and Boethel 1986; Harvey et al. 2003, 2007c) trophic level. On the other hand, prolonged development of the host on poor quality host plants may extend the window of vulnerability to parasitism (the “slow-growth-high-mortality hypothesis”, *sensu* Benrey and Denno 1997; Williams 1999). These results suggest that top-down and bottom-up control of insect herbivores may not be simple alternatives and that we may need to acknowledge complex interactions between top-down and bottom-up effects that vary over spatial and temporal gradients (Gripenberg and Roslin 2007).

Generalist insects are generally much more strongly affected by plant secondary chemistry than specialists (Blau et al. 1978; Giamoustaris and Mithen 1995). Therefore, to better understand the effects of inter-population variation in plant

defences on higher trophic level interactions, these interactions should be studied for generalists and specialists separately. Because some defensive traits are inducible (see Karban and Baldwin 1997) induction patterns of defensive traits in response to specialist and generalist herbivores should also be considered.

Populations of wild cabbage, *B. oleracea* L. (Brassicaceae) grow naturally along the Atlantic coastlines of the United Kingdom and France (Mitchell and Richards 1979). Previous studies have reported differences in the expression of glucosinolates (hereafter GS) among populations of *B. oleracea* over a limited spatial scale (Mithen et al. 1995; Moyes et al. 2000; Moyes and Raybould 2001). The expression of GS is under control of several genes (Magrath et al. 1993; Halkier and Gershenzon 2006) and is highly heritable (Mithen et al. 1995). Raybould et al. (1999) have demonstrated that the Dorset populations of wild *B. oleracea* are genetically differentiated. Since these populations have evolved under natural selection pressures, differences between the populations are most likely caused by variation in local biotic and abiotic characteristics. For instance, some of the Dorset populations of *B. oleracea* are found on exposed cliff sides, whereas others grow in more secluded areas. This profoundly affects their degree of exposure to the prevailing wind, which in turn may hinder the ability of flying insects to either colonise the plants, or else influences the dispersal of plant-related odours that are used by insects to locate plants amongst other stands of interstitial vegetation. However, random processes, such as genetic drift, may also play a role in shaping differences in GS chemistry.

Herbivore densities on different populations of wild *B. oleracea* in Dorset have been shown to vary, although, densities of specialist herbivores were not correlated with measured levels of GS compounds (Moyes et al. 2000). In the study by Moyes et al. (2000), herbivore densities (higher trophic levels were not included in the survey) were measured over two consecutive years, but samples for GS were only taken once. GS levels have been shown to fluctuate over the course of a growing season and tend to be higher in the fall than in the summer (Agerbirk et al. 2001; Gols et al. 2007). Furthermore, GS concentrations change in response to herbivore feeding damage (Bodnaryk 1992; Agrawal et al. 2002; Traw and Dawson 2002). Therefore, GS concentrations in natural populations are the combined result of constitutive and induced levels as well as seasonal fluctuations. To link levels of GS to performance of herbivores in the field, both constitutive and induced levels of GS need to be separated (but see Traw 2002).

This study examines growth and development of two herbivorous insect species, *Pieris rapae* L. (Lepidoptera, Pieridae) and *Mamestra brassicae* L. (Lepidoptera, Noctuidae), when reared on three of the wild Dorset populations and a cultivar of *B.*

oleracea under controlled glasshouse conditions. Both herbivore species are amongst the most serious pests of cultivated cabbage over much of Europe, but may also attack other related wild crucifers (Theunissen et al. 1985). The cultivar served as a population with low levels of GS in leaf tissue compared to the wild populations. *P. rapae* is a specialist herbivore on brassicaceous plant species, whereas *M. brassicae* is a generalist insect herbivore, which feeds on plants in several plant families including the Brassicaceae (Carter 1984). Furthermore, we investigated the effect of food plant quality mediated through the host on the performance of two closely related solitary endoparasitoids of the two herbivores, *Cotesia rubecula* Marshall (Hymenoptera: Braconidae) and *Microplitis mediator* Halliday (Hymenoptera: Braconidae). In addition we measured levels of GS in plants damaged by herbivores and determined if they correlated with insect performance. We predict that differences in GS levels among the different *B. oleracea* populations are reflected in the development and survival of the insect herbivores and their parasitoids, but that the development of the generalist and its parasitoid are more negatively affected than the better adapted specialist and its parasitoid.

Methods

Plants

Brassica oleracea seeds were collected from several plants (>15) in three wild populations growing within 10 km distance on chalk cliffs along the south coast of Great Britain, near Swanage in Dorset, in September 2005. The three wild populations were located at sites known as 'Old Harry' (50°38'N, 1°55'E), 'Kimmeridge' (50°35'N, 2°03'E), and 'Winspit' (50°36'N, 2°07'E). Hereafter the different populations will be referred to as OH (Old Harry), KIM (Kimmeridge) and WIN (Winspit). As a reference plant population, we used a cultivated variety of *B. oleracea*, gemmifera (Brussels sprout) cv. Cyrus (hereafter CYR), because it contains low levels of GS compared to the wild Dorset populations of *B. oleracea* in England (Moyes et al. 2000; Gols et al. 2007).

Seeds were germinated and seedlings were subsequently transferred to 2-l pots containing a soil mixture of 30% sand, 5% clay and 65% peat. Plants were grown in a greenhouse at 20-30 °C, 40-80% r.h, and a photoperiod of at least 16 h. If the light dropped below 500 $\mu\text{mol photons/m}^2/\text{s}$ during the 16-h photoperiod, supplementary illumination was applied by high-pressure mercury lamps. Plants were watered daily. When the plants were 4 weeks old, they were fertilised once a week with 100 ml 0.5 Hoagland solution, which was applied to the soil.

Plants were 8 weeks old when they were used in experiments. Fertilising and watering were continued during the experiments.

Insects

The insect herbivores and parasitoids used in this study are native to the Palaearctic region and sympatric in their overall distributions. All insects, with the exception of *Mi. mediator*, were originally collected from a Brussels sprout field in the vicinity of Wageningen, The Netherlands and were maintained on Brussels sprouts in the laboratory. Cocoons of *Mi. mediator* were obtained from a culture maintained at Agriculture Canada Laboratories in Saskatoon, Saskatchewan, Canada. *C. rubecula* is a highly specialised solitary endoparasitoid that only attacks first and second instar larvae (hereafter L1-L2) of *P. rapae* (Harvey et al. 1999). Parasitised host larvae were reared on Brussels sprout until the parasitoids had completed larval development and egressed from the host. *Mi. mediator* is a fairly specialised solitary endoparasitoid that attacks L1-L4 instar larvae of *M. brassicae* and a few closely related hosts in the moth family Noctuidae (Arthur and Mason 1986). In this study *Mi. mediator* was reared on *M. brassicae*. Newly hatched L1 instar larvae obtained from the general *M. brassicae* culture were reared on an artificial insect diet (ingredients per 1 l diet: 28 g agar, 160 g corn flower, 50 g beer yeast, 50 g wheat germs, 2 g sorbic acid, 1.6 g methyl-4-hydroxybenzoate, 8 g ascorbic acid, 0.5 g streptomycin and water). When the larvae had developed into L2, they were parasitised by mated *Mi. mediator* females in large Petri dishes. Parasitised caterpillars were reared on artificial diet until the parasitoid larvae egressed from the host and pupated. Parasitoid cocoons were collected and transferred to a new cage (*C. rubecula*) or Petri dish (*Mi. mediator*). Newly emerged adults were provided with honey and water and mated females were used in experiments approximately 5 days after adult emergence. All insects used in this study were reared in climate rooms or greenhouses at 22 ± 2 °C, 50-70% r.h, and a photoperiod of at least 16 h.

Glucosinolate analysis

To determine GS levels in leaf tissues of the different *B. oleracea* populations, samples were taken from undamaged control plants ($n = 5$) and from *P. rapae*-damaged plants whether the caterpillars were either parasitised ($n = 9$) or unparasitised ($n = 6$). The plants that were used in the performance bioassay (see *Insect performance*) were also used for tissue sampling. Samples were taken from plants that had been damaged by *P. rapae* caterpillars for 7 days. Undamaged control plants were of the same age and were kept in the same greenhouse. A second cohort of undamaged plants was sampled two weeks later to establish the relationship between GS content and survival of *M.*

brassicae (see statistical analysis). Two discs, 7 mm in diameter, were punched per leaf, one from each central part of a leaf half, and six fully unfolded leaves were sampled per plant. Leaf discs were pooled per plant in 2-ml Eppendorf vials and frozen in liquid nitrogen immediately after sampling. Samples were later freeze dried and pulverised. Fifty mg aliquots of dried material were weighed in 2 ml centrifuge tubes. GS were extracted and purified as described in van Dam et al. (2004), which should be consulted for a more detailed description. GS were separated on a reverse phase C-18 column (Alltima C-18, 3µm, 150 x 4.6 mm, Alltech, Deerfield, IL, USA) on HPLC (DIONEX, Sunnyvale, CA, USA) with an acetonitrile water gradient. Detection was performed with a DIONEX PDA-100 Photodiode array detector set to scan from 200 to 350 nm. For quantification, sinigrin (Sigma, St. Louis, MO, USA) was used as an external standard. Peaks were integrated at 229 nm for which standard response factors have been defined (EC 1990). The different GS were identified based on their retention times and UV spectra compared to those of the pure compounds, sinigrin (Sigma, St. Louis, MO, USA) and glucobrassicin (kindly provided by M. Reichelt, MPI for Chemical Ecology, Jena, Germany), or compared with a certified oil seed reference (EC Community Bureau of Reference BCR-367R, Fluka, Buchs, Switzerland).

Insect performance

To investigate whether variation in plant chemistry affects higher trophic levels, the different insect herbivores and their parasitoids were reared on plants from the four different *B. oleracea* populations. Experiments were conducted in two greenhouses under the same conditions as for the plants. We used a separate greenhouse for each herbivore-parasitoid combination.

Specialist plant association: *Pieris rapae* and *Cotesia rubecula*. To obtain plants with eggs of *P. rapae*, one plant from each population was placed in the rearing cage with adult *P. rapae* butterflies for 4 h. Plants were then removed from the cage and left in the rearing room for the eggs to hatch and the larvae to develop until they reached L2. For each plant population one cohort of 54 unparasitised larvae, which served as a control, was transferred and divided over 6 new plants of each population. A second cohort of 100 larvae was parasitised by *C. rubecula*. For parasitism, an individual mated female parasitoid was presented with a single L2 *P. rapae* larva. A larva was considered to be parasitised when the female inserted into and removed her ovipositor from the host. Each parasitoid female was allowed to parasitise up to a maximum of 10 larvae. Parasitised larvae were evenly distributed over 9 new plants of the population on which the unparasitised larvae had been feeding previously.

Caterpillars were allowed to develop and move freely

on the plants until pupation of the herbivore or egression of the parasitoid larva from the host and subsequent pupation. Unparasitised caterpillars tend to wander and leave the food plant in the final phase of the larval development to search for a site to pupate. Therefore, unparasitised control larvae in the late final (fifth) instar were transferred to plastic containers (15 x 12 x 6 cm) containing some excised leaf material. Larvae were checked twice a day and when they had pupated, the date of pupation was recorded and the pupae were weighed on a Cahn microbalance (Cahn Instruments, Cerritos CA., USA, accuracy 1 μ g).

Parasitoid cocoons were collected from the plant and kept individually in labelled vials until adult emergence. When the adult wasps emerged, the date of eclosion was recorded and their sex was determined visually (the ovipositor of female wasps is visible on the abdomen). Wasps were anaesthetised with CO₂ and subsequently weighed on the Cahn microbalance. A subsample of male and female *C. rubecula* wasps were dried in an oven at 80 °C for three days to establish the relationship between fresh and dry mass. Cocoons were checked several times daily for adult parasitoid emergence. As the time of oviposition, we used the time at which 50% of the larvae were parasitised, which took about 1 h. Since the time of pupation of the herbivore could not be determined accurately, development time was measured in days.

Generalist plant association: *Mamestra brassicae* and *Microplitis mediator*. Sheets with *M. brassicae* eggs were obtained from the general culture. These sheets were incubated until the eggs hatched. Newly hatched larvae were then evenly distributed among four plants, one plant of each *B. oleracea* population. When the larvae had reached L2, they were parasitised by *Mi. mediator*, using the method described above for *C. rubecula* and *P. rapae*. Unfortunately, the number of *M. brassicae* larvae surviving on the different plant populations varied greatly. The highest number of larvae was recovered from CYR and KIM plants. In contrast, none of the larvae survived on WIN, which was therefore excluded from the parasitism experiment. To obtain sufficient larvae that were reared on OH, we transferred some of the larvae that were initially reared on CYR to the OH population after parasitism. The final number of parasitised hosts on the different populations was 70 larvae on CYR and 100 larvae on both KIM and OH. To ensure that the larvae did not leave their respective food plants, parasitised larvae were reared in plastic containers (15 x 12 x 6 cm) on excised leaves. Every two days survival was monitored, leaf material replaced by fresh leaves, and feces removed until the parasitoid egressed from the host. Parasitoid cocoons were collected and transferred to labelled glass vials. Development time and adult fresh mass was

determined as for *C. rubecula*.

Because early survival of the *M. brassicae* larvae varied considerably when reared on intact plants from the different populations, development of healthy (= unparasitised) larvae was monitored in more detail in a separate experiment. Newly hatched larvae, (200 per plant population), were transferred in groups of 10 to small Petri dishes (9 cm diameter) with pieces of excised leaves. Every two days survival was monitored, leaf material replaced with fresh leaves, and feces removed. As the larvae grew and molted to L3, they were moved to larger Petri dishes (13 cm diameter) and, in the final instar (L5), to plastic containers (15 x 12 x 6 cm). To reduce the number of plants required, the number of individuals was reduced to approximately 100 on CYR, KIM and OH plants when the caterpillars reached L3. At this point, the number of remaining caterpillars on WIN plants was already less than 100 individuals. Petri dishes were randomly selected from each population treatment and discarded. When the caterpillars reached L5, containers were lined with a mixture of soil and vermiculite in preparation for pupation, which in *M. brassicae* occurs belowground. This took approximately one week. Pupae were collected daily and their fresh mass measured immediately.

Statistical analysis

Egg-to-pupa development time and pupal mass of individual *P. rapae* and *M. brassicae* reared on the different plant populations were analysed using a general linear model ANOVA with plant population as main factor. Similarly, we used a general linear model ANOVA to compare egg-to-adult development time and adult body mass of individual *C. rubecula*. Here we included plant population and sex as well as their interaction as factors in the model. To compare levels of GS a general linear model ANOVA was used on ln transformed GS concentrations in individual plants with plant population, plant treatment (undamaged control, damaged by unparasitised *P. rapae* and damaged by parasitised *P. rapae*) and plant population*treatment interaction as factors. Post-hoc Tukey-Kramer multiple comparisons tests were conducted when the ANOVA models were significant. Analyses were carried out using SAS software package 8.02 (1999-2001 SAS Institute Inc).

Data on the survival of *M. brassicae* were analyzed using non-parametric Kaplan-Meier survival curves (Kleinbaum 1996). Data were censored to account for the reduction in number of larvae on plants. The log-rank test was used to compare the survival curves on the different plant populations and a sequential Bonferroni correction was applied to adjust α for multiple comparisons.

Linear least square regression analysis was carried out to establish the relationship between mean concentrations of GS

compounds in the different plant populations and mean values of the different fitness parameters (development time, adult body mass, survival) of the herbivores when reared on these plant populations. For the analysis, we used GS concentrations measured in plant tissue when the larvae were in the third larval instar. *Mamestra brassicae* was reared on detached leaves; therefore, we used GS data of undamaged plants that were sampled in the third week since oviposition. For *P. rapae* we used the GS concentrations measured in plants that were fed upon by *P. rapae* for 7 days.

Results

Glucosinolates

GS analyses of samples taken from leaf tissues revealed considerable quantitative variation between the four *B. oleracea* populations (Fig. 1 and 2). In all plant populations the same nine different GS were detected except for neoglucobrassicin in

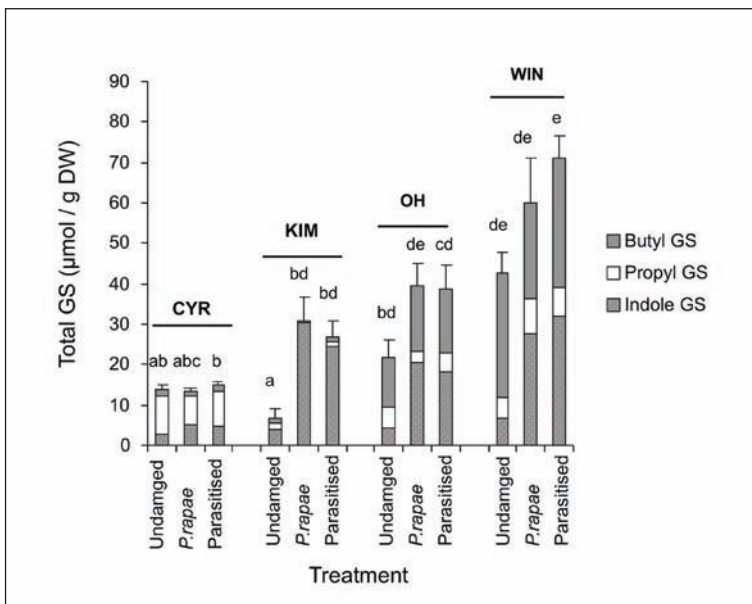
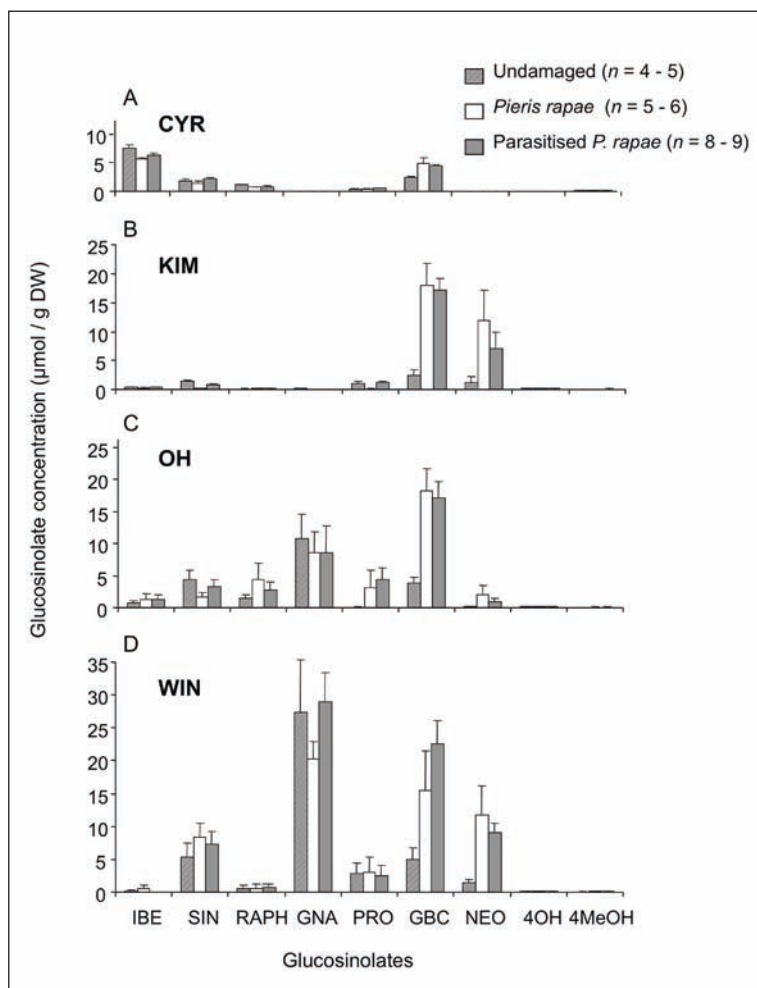


Figure 1: Total glucosinolate (GS) concentrations in leaf tissues (mean + SE; $\mu\text{mol/g DW}$) in a cultivar (CYR) and three wild populations (KIM, OH, and WIN) of *Brassica oleracea* from the UK (Dorset), when reared under standardised greenhouse conditions. GS quantities were measured in leaf tissue sampled from undamaged plants, plants damaged by unparasitised *Pieris rapae* larvae and plants damaged by *P. rapae* larvae that were parasitised by *Cotesia rubecula*. Total GS levels were divided into aliphatic GS derived from a propyl (IBE and SIN, see Fig. 2 for the full names) and a butyl side chain (RAPH, GNA and PRO), and indole GS (GBC, NEO, 4OH, 4MeOH). Bars with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means with $\alpha = 0.05$)

CYR plants; however, previous work detected low levels of this compound in the same cultivar (Gols et al. 2007). The following GS were found: two propyl-derived GS (glucoiberin and sinigrin), three butyl-derived GS (progoitrin, gluconapin, and glucoraphanin) and four indole GS (glucobrassicin, neoglucobrassicin, 4-hydroxyglucobrassicin, and 4-methoxyglucobrassicin). Plant population had a significant effect on levels of all individual GS except for progoitrin ($F_{3,62} = 1.75$, $P = 0.17$) and

Figure 2. Glucosinolate (GS) concentrations in leaf tissues (mean + SE; $\mu\text{mol/g DW}$) in (A) a cultivar (CYR), and in three different wild populations, (B) KIM, (C) OH, and (D) WIN, of *Brassica oleracea* from the UK (Dorset), when reared under standardised greenhouse conditions. GS quantities were measured in leaf tissue sampled from undamaged plants (hatched bars), plants damaged by unparasitised *Pieris rapae* larvae (open bars) and plants damaged by parasitised *P. rapae* larvae (solid bars). Damaged plants were exposed to herbivore feeding for seven days and all plants were of the same age when sampled. The following GS were detected: glucoiberin (IBE), sinigrin (SIN), glucoraphanin (RAPH), gluconapin (GNA), progoitrin (PRO), glucobrassicin (GBC), neoglucobrassicin (NEO), 4-hydroxy glucobrassicin (4OH), and 4-methoxy glucobrassicin (4MeOH).



4-hydroxyglucobrassicin ($F_{3,62} = 1.76$, $P = 0.16$).

Larval feeding damage had a significant effect on concentrations of neoglucobrassicin ($F_{2,62} = 19.8$, $P < 0.001$) and glucobrassicin ($F_{2,62} = 32.0$, $P < 0.001$), but not on the other GS. For glucobrassicin the interaction between plant population and treatment was also significant ($F_{6,62} = 2.57$, $P = 0.03$). Only in the wild plant populations were glucobrassicin concentrations higher in damaged plants than in undamaged plants. In WIN plants the difference between damaged and undamaged plants was only significant for plants damaged by parasitised *P. rapae* caterpillars. The effect of induction was most pronounced in the KIM population, where damage resulted in a 4-fold increase of total GS, whereas there was little change in the CYR plants (Fig. 1). Parasitised and unparasitised caterpillars induced the plants in a similar way (pairwise comparisons were never significantly different).

The relative contribution of the different GS to the total

GS profile differed among populations and between damaged and undamaged plants (Fig. 1 and 2). In undamaged plants, glucoiberin was the dominant compound in CYR plants (54% of total), whereas in OH and WIN the GS profile was dominated by gluconapin (50% and 64% of total, respectively). In undamaged KIM plants, neo-glucobrassicin and sinigrin comprised 20% and 17% of the GS content, respectively. In plants that were induced by unparasitised or parasitised *P. rapae* indole GS dominated the GS profile. In the KIM plants, indole GS contributed >90% of the total GS content in induced plants, compared to between 39% and 52% in the other three populations.

Performance studies

Pieris rapae - *Cotesia rubecula* All plant populations were readily accepted for oviposition by female *P. rapae* butterflies. Females laid more than 150 eggs on each plant from the four different populations within a 4 h oviposition period. Plant population had a significant effect on pupal mass ($F_{3,183} = 5.0$, $P = 0.002$; Fig. 3A) and egg-to-pupa development time ($F_{3,183} = 44.6$, $P < 0.001$; Fig. 3B). Pupal mass was significantly higher when the larvae were

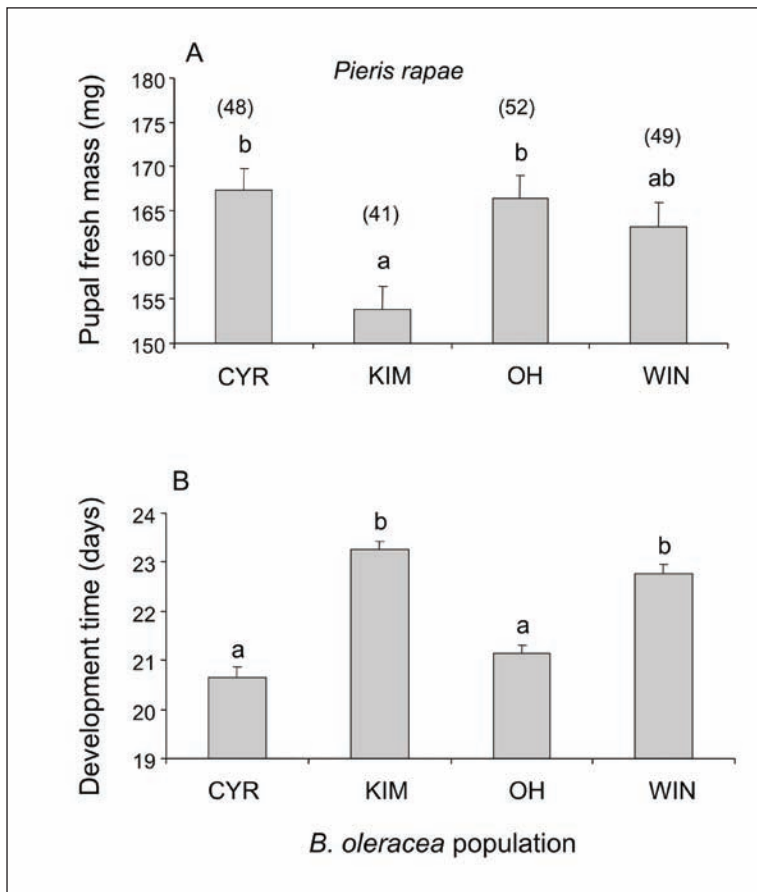


Figure 3: Pupal fresh mass (A), and development time from egg to pupa (B) of *Pieris rapae* that were reared in a greenhouse on either a cultivar (CYR) or on one of three different wild populations (KIM, OH, and WIN) of *Brassica oleracea* from the UK (Dorset). Values are means $\pm$ SE; bars with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means with $\alpha = 0.05$). Numbers between brackets denote number of individuals (n).

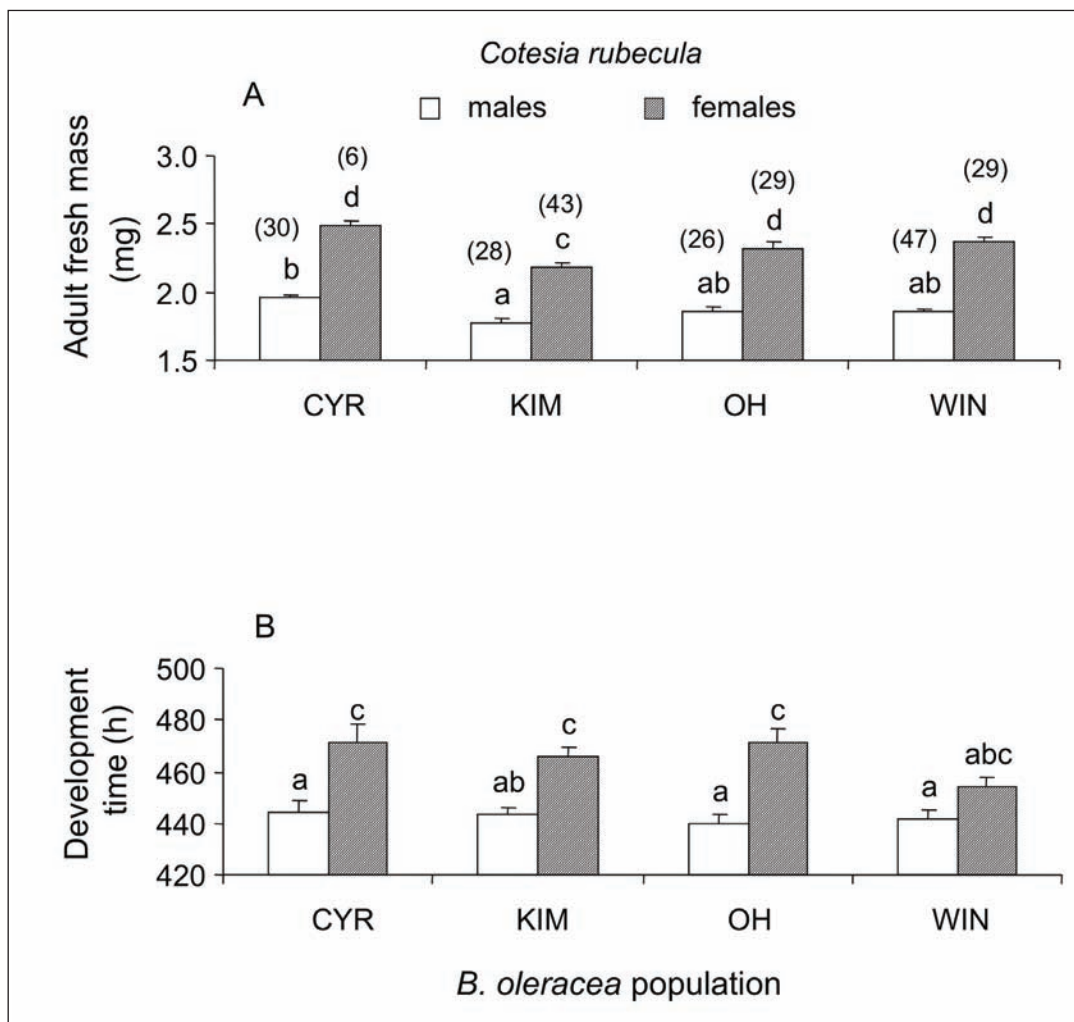


Figure 4: Adult fresh mass (A), and development time from egg to adult (B) of *Cotesia rubecula* that were reared on *Pieris rapae* in a greenhouse on either a cultivar (CYR) or on one of three different wild populations (KIM, OH, and WIN) of *Brassica oleracea* from the UK (Dorset). Results are given separately for males (with bars) and females (dashed bars). Values are means $\pm$ SE; bars with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means with $\alpha = 0.05$). Numbers between brackets denote the number of individuals (n).

reared on CYR and OH compared to KIM. Pupae reared on WIN plants were of intermediate mass. Larvae developed the fastest on the populations on which they attained the highest pupal mass, i.e. CYR and OH (Fig. 3). The egg-to-pupa development time was longest on KIM and WIN plants (Fig. 3B), and was about 2.5 days longer than on OH and CYR plants.

Plant population had also a significant effect on adult dry mass of the parasitoid, *C. rubecula*, ($F_{3,230} = 13.9$, $P < 0.001$; Fig. 4A), but not on egg-to-adult development time ($F_{3,230} = 1.89$, $P = 0.13$; Fig. 4B). Wasps, both males and females, emerging from *P. rapae* caterpillars reared on CYR plants were heavier than wasps reared on the KIM population. Adult body mass of wasps reared on OH and WIN plants was not significantly different of wasps reared on either CYR or KIM plants (Fig. 4A). The relationship between fresh mass and dry mass of adult wasps was highly significant (males $F_{1,21} = 438$, $P < 0.001$, $r^2 =$

0.95; females, $F_{1,32} = 368$, $P < 0.001$, $r^2 = 0.92$). Thus, fresh mass accurately approximates body mass of *C. rubecula*. Furthermore, males were smaller ($F_{1,230} = 332$, $P < 0.001$; Fig. 4A) and developed faster ($F_{1,230} = 47.7$, $P < 0.001$; Fig 4B) than female wasps.

Mamestra brassicae - *Microplitis mediator* Survival of the generalist herbivore, *M. brassicae*, was greatly affected by plant population (Fig. 5). Individual survival curves were all significantly different from each other except for the CYR and KIM curves. None of the larvae reared on the WIN population survived, whereas about 60% of the larvae reared on OH and about 80% of the larvae reared on CYR and KIM plants successfully pupated. Many larvae disappeared during the bioassay. We assumed that these larvae had been cannibalised, since dead ones were also found; furthermore, the larvae could not escape from the Petri dishes. Mortality due to cannibalism was much higher on plants of poor food quality. On OH and WIN plants, 17.5 % and 57.3% of the larvae died due to cannibalism, whereas this rate was less than 2% on CYR and KIM plants.

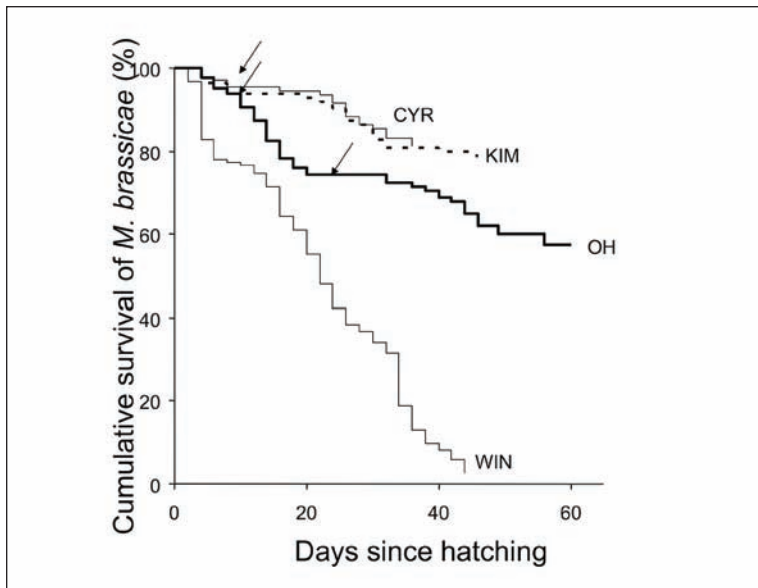
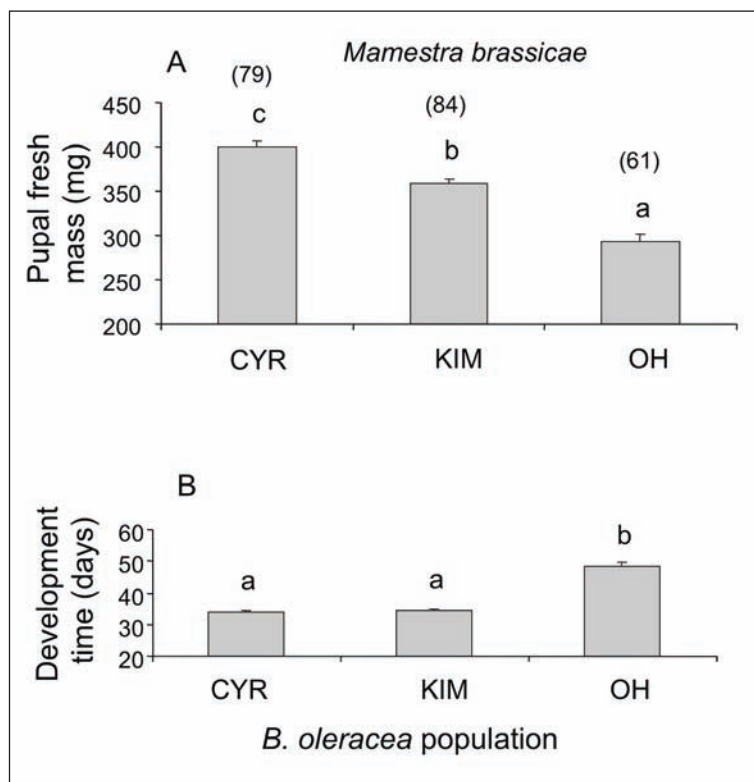


Figure 5: Cumulative survival tendencies of *Mamestra brassicae* larvae when reared on detached leaves from either a cultivar (CYR) or on one of three different wild populations (KIM, OH, and WIN) of *Brassica oleracea* from the UK (Dorset). The arrows indicate the time point at which the numbers of larvae were reduced and the analysis was adjusted accordingly (censored data). All pairwise comparisons (log-rank test with Bonferroni corrected α 's) of survival curves were significantly different except for the CYR-KIM comparison ($P = 0.76$). For other pairwise comparisons: CYR-OH, $P = 0.02$; OH-KIM, $P = 0.008$; WIN-KIM, $P < 0.001$; WIN-OH, $P < 0.001$; WIN-CV, $P < 0.001$).

Plant population also had a significant effect on pupal biomass ($F_{2,221} = 66.0$, $P < 0.001$; Fig. 6A) as well as egg-to-pupa development time ($F_{2,221} = 156$, $P < 0.001$; Fig 6B). Pupal weights were highest when the larvae were reared on CYR plants, intermediate when reared on KIM plants and lowest when reared on OH plants (Fig. 6A). Larval development rates were equivalent on CYR and KIM, but caterpillars took 14 days longer to develop into pupae on OH (Fig. 6B).

Figure 6: Pupal fresh mass (A), and development time from egg to pupa (B) of *Mamestra brassicae* that were reared in a greenhouse on either a cultivar (CYR) or on one of three different wild populations (KIM, OH, and WIN) of *Brassica oleracea* from the UK (Dorset). No bars are provided for the WIN population, because all larvae perished before pupation. Values are means $\pm$ SE; bars with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means with $\alpha = 0.05$). Numbers between brackets denote number of individuals (n).



As in the herbivore, survival of its parasitoid, *Mi. mediator*, was greatly affected by plant population. Since almost all first instar caterpillars died when reared on intact WIN plants, this population was not used for experiments with *Mi. mediator*. The population on which the host developed had a significant effect on survival of the parasitoids ($\chi^2=7.19$, d.f. = 2, $P = 0.03$). Survival of *Mi. mediator* was the highest, i.e. 21.6%, when the host, *M. brassicae*, was reared on CYR plants. Survival was 16% on KIM, whereas only 4% of the parasitised larvae developed into adults on OH. Since the number of surviving adult wasps was very low, especially the number of females, data on development time and biomass are not presented.

Regression analyses of glucosinolate concentrations and herbivore development

Regression analyses revealed only a significant positive linear relationship between neoglucobrassicin concentration and development time of *P. rapae* (Fig. 7A). For *M. brassicae*, a significant negative relationship was found between concentrations of gluconapin, sinigrin and total GS, respectively, and survival of the larvae (Fig. 7B-D).

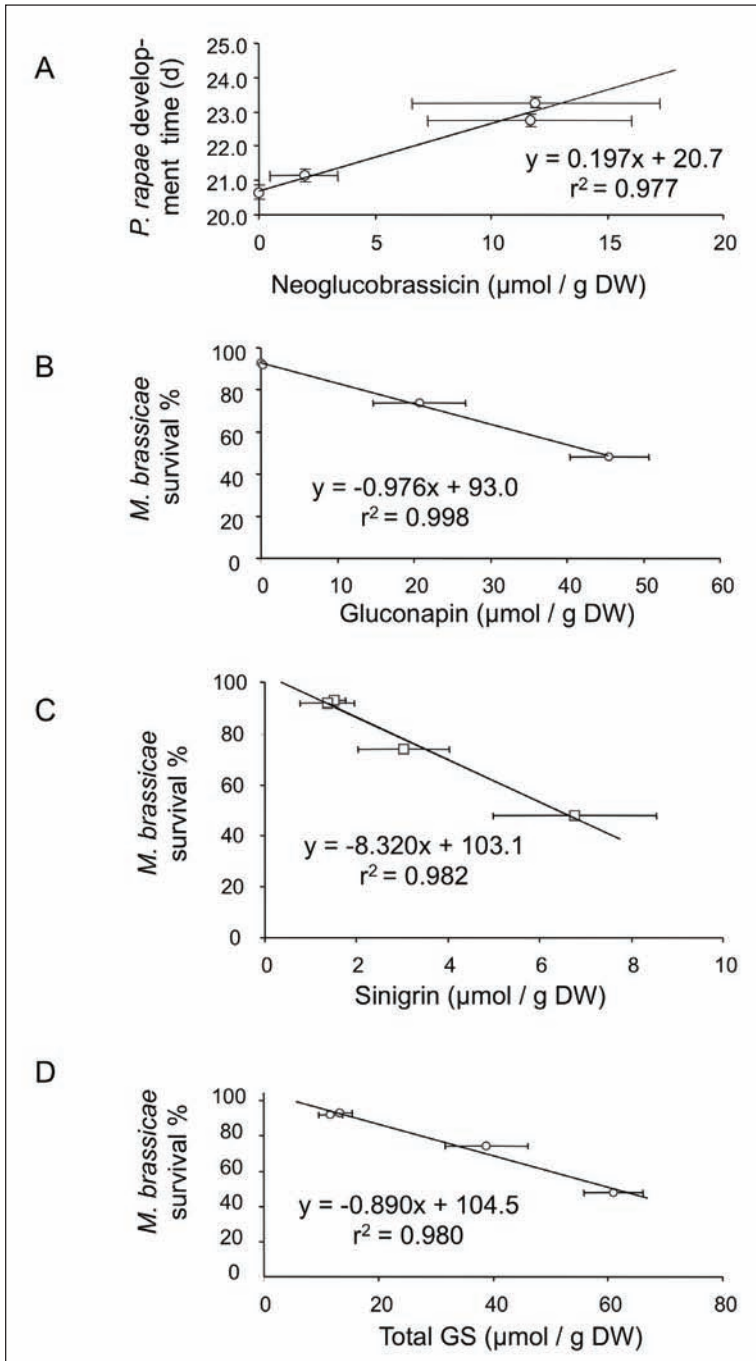


Figure 7: Relationship between mean concentrations of three GS compounds and for total GS from four different *Brassica oleracea* populations and mean fitness parameters of two herbivores, *Pieris rapae* (A) and *Mamestra brassicae* (B-D). A significant linear relationship was found for neoglucobrassicin ($F_{1,2} = 84.1$, $P = 0.01$) and *P. rapae* development time, as well as for gluconapin ($F_{1,2} = 1150$, $P < 0.001$), sinigrin ($F_{1,2} = 112$, $P = 0.009$) and total GS ($F_{1,2} = 100$, $P = 0.01$), respectively, and survival of *M. brassicae*. GS concentrations (mean $\pm$ SE) were measured halfway through the larval development of the herbivores. For *P. rapae*, which was reared on intact plants, GS concentrations were based on samples ($n = 5$) taken from plants from each population that had been fed upon by *P. rapae* caterpillars for seven days. *M. brassicae* was reared on detached leaves; therefore, GS concentrations for *M. brassicae* were determined in undamaged plants ($n = 5$) that were sampled three weeks after oviposition. For survival of *M. brassicae* we used the percentage of caterpillars that were still alive at day 22 on each of the four plant populations. Least square regression equations are given in the graph.

Discussion

Glucosinolate levels in different populations of *Brassica oleracea*

The results of this study show that there was significant variation in GS levels in plants from three wild populations and one cultivar of *B. oleracea* reared under standardised laboratory conditions. Earlier work similarly reported significant differences in GS concentrations amongst several different wild populations of this species from the same sites (Mithen et al. 1995; Moyes et al. 2000). Previous studies with the WIN and KIM populations (Mithen et al. 1995; Moyes et al. 2000) reported higher levels of aliphatic GS in leaf tissues collected directly from plants in the field than we observed in greenhouse reared plants. Levels of the indole GS in those studies were similar to the levels we recorded in herbivore-damaged plants. The differences in GS patterns between the two populations were the same, i.e. the KIM population produced much lower concentrations of aliphatic GS than the WIN population. The observed differences in chemical profiles between plants in the studies by Mithen et al. (1995), Moyes et al. (2000) and this study may be due to concomitant differences in ambient conditions in which the plants were grown (field versus greenhouse). Furthermore, the field plants may have been older than the plants used here, bearing in mind that some of the *B. oleracea* plants in the Dorset populations exceed 20 years of age (Mitchell and Richards 1979) and GS levels decline with leaf age (Shelton 2005; Gols et al. 2007).

Feeding damage from *P. rapae* differentially affected levels of individual GS. Concentrations of the two indole GS, glucobrassicin and neoglucobrassicin, increased dramatically in response to *P. rapae* feeding on the wild populations, whereas levels of aliphatic GS remained the same as in undamaged plants. Different biosynthetic pathways are involved in the production of aliphatic and indole GS (Halkier and Gershenzon 2006), which may explain the differential effect of herbivore feeding on the expression of these GS. Fatouros et al. (2005) reported that CYR plants treated with regurgitant of parasitised *P. rapae* emitted a different volatile blend than plants treated with regurgitant of unparasitised caterpillars. We showed that the expression of GS did not differ between plants damaged by parasitised and unparasitised *P. rapae*.

Insect development on different *Brassica oleracea* populations

We found that the development of the insect herbivores differed significantly across the different populations of *B. oleracea*. The development of the specialist herbivore *P. rapae* was much less negatively affected than that of the generalist herbivore *M.*

brassicae. Specialist herbivores of plants in the Brassicaceae, such as *P. rapae*, have evolved a specific detoxification system to convert the production of deleterious GS breakdown products such as isothiocyanates into less harmful compounds (Ratzka et al. 2002; Wittstock et al. 2004). For example, *P. rapae* produces an enzyme, a so-called nitrile-specifier protein, which redirects the hydrolysis pathway of GS from isothiocyanates into the production of less toxic nitriles (Wittstock et al. 2004). Here we show that the performance of *P. rapae* was differentially affected by *B. oleracea* populations that differ in their GS profile. Regression analyses revealed that *P. rapae* developed most poorly on plants from the KIM and WIN population, which contained the highest levels of the indole GS, neoglucobrassicin. These two populations differed considerably with respect to other GS concentrations, suggesting that neoglucobrassicin affects plant quality for this herbivore species and that detoxification of this GS may be less efficient.

The role of indole GS in defence against insect herbivory is poorly understood, in part, because levels of indole GS in foliage of cultivated *B. oleracea* and other Brassicaceous species are much lower than in wild *B. oleracea* plants (Giamoustaris and Mithen 1995; Rosa 1999; Traw 2002). Only the hydrolysis products of GS, such as isothiocyanates, have been shown to be active against insects (Agrawal and Kurashige 2003; Wittstock et al. 2004). These products are formed after tissue damage, followed by myrosinase enzyme activity. Hydrolysis of aliphatic GS results in the formation of stable isothiocyanates. Attempts to isolate stable isothiocyanate products of indole GS have not been successful (Bones and Rossiter 2006) suggesting that hydrolysis products of indole GS undergo further degradation. Whether indole GS and their breakdown products are detoxified by insect herbivores specialised on the Brassicaceae in the same way as aliphatic GS is unknown and merits further investigation.

For *M. brassicae*, regression analyses revealed a significant negative relationship between concentrations of the two aliphatic GS, sinigrin and gluconapin, and herbivore survival. *M. brassicae* developed most poorly on WIN and OH plants, which contained not only high levels of sinigrin and gluconapin, but also high overall GS levels. We also found a negative relationship between total levels of GS and survival of *M. brassicae* larvae, suggesting that *M. brassicae* responds to high levels of GS rather than to specific GS. Furthermore, *M. brassicae* was reared on detached leaves, whereas *P. rapae* was reared on intact plants. Further research is necessary to reveal the time course of GS induction. The induction of indole GS may not have reached similar levels in detached leaves as in intact plants and this may have influenced the development of *M. brassicae*.

The development of the two parasitoid species also differed across the various populations of *B. oleracea*. Most importantly, parasitoid development and survival appeared to

closely reflect the effects of plant quality on host development. Various studies have similarly reported that the performance of both herbivores and their natural enemies are negatively affected by plant allelochemicals (Campbell and Duffey 1979; Barbosa et al. 1986; Gunasena et al. 1990; Sznajder and Harvey 2003; Harvey 2005, 2007c).

Little is known about the means by which parasitoid larvae deal with allelochemicals in host diet that may diffuse through the gut wall and accumulate in host haemolymph or fat body. Endoparasitoids may be particularly susceptible to plant toxins because the alimentary tract of parasitoid larvae is not externally connected until after the parasitoid disposes of host tissues and constructs a cocoon, into which the meconium is voided (Quicke 1997). Barbosa et al. (1986) and Bowers (2003) found traces of nicotine and catalpol in the larval by-products (cocoon silk, meconium) of the parasitoid *Cotesia congregata* developing in two of its hosts, *Manduca sexta* and *Ceratomia catalpae*, respectively. It is likely that the ability of parasitoids to deal with plant allelochemicals is largely dependent on the degree to which they are exposed to them, and suggests that species such as *C. rubecula* probably are well adapted to deal with high levels of GS in host diet (see also Harvey et al. 2003).

Evolution of plant defences in natural populations of *Brassica oleracea*

Although plant defences have been well-studied in a multitrophic framework, and the effects of secondary chemistry on herbivores and their natural enemies are well documented (see reviews by Harvey 2005; Ode 2006), the factors underlying the genetic variation in plant defence in natural populations are virtually unknown. Several studies have investigated spatial and temporal variation in plant chemistry over broad geographical scales (Agrawal 2005; Zangerl and Berenbaum 2005). The cabbage populations studied here all grow within 15 km of one another, and perhaps more striking is the fact that the two most different populations in terms of defence chemistry (WIN and KIM) are separated by less than 5 km. Notably, the KIM plants grow atop cliffs that are fully exposed to strong prevailing westerly winds, whereas the WIN population occurs in a very sheltered valley. Although they have not been measured, it is almost certain that important microclimatic variables, such as wind speed, temperature, and access to light and water differ markedly between the different coastal populations of *B. oleracea*. This variation in abiotic traits may be linked with biotic selection pressures, such as herbivory and the efficacy of natural enemies, such as parasitoids.

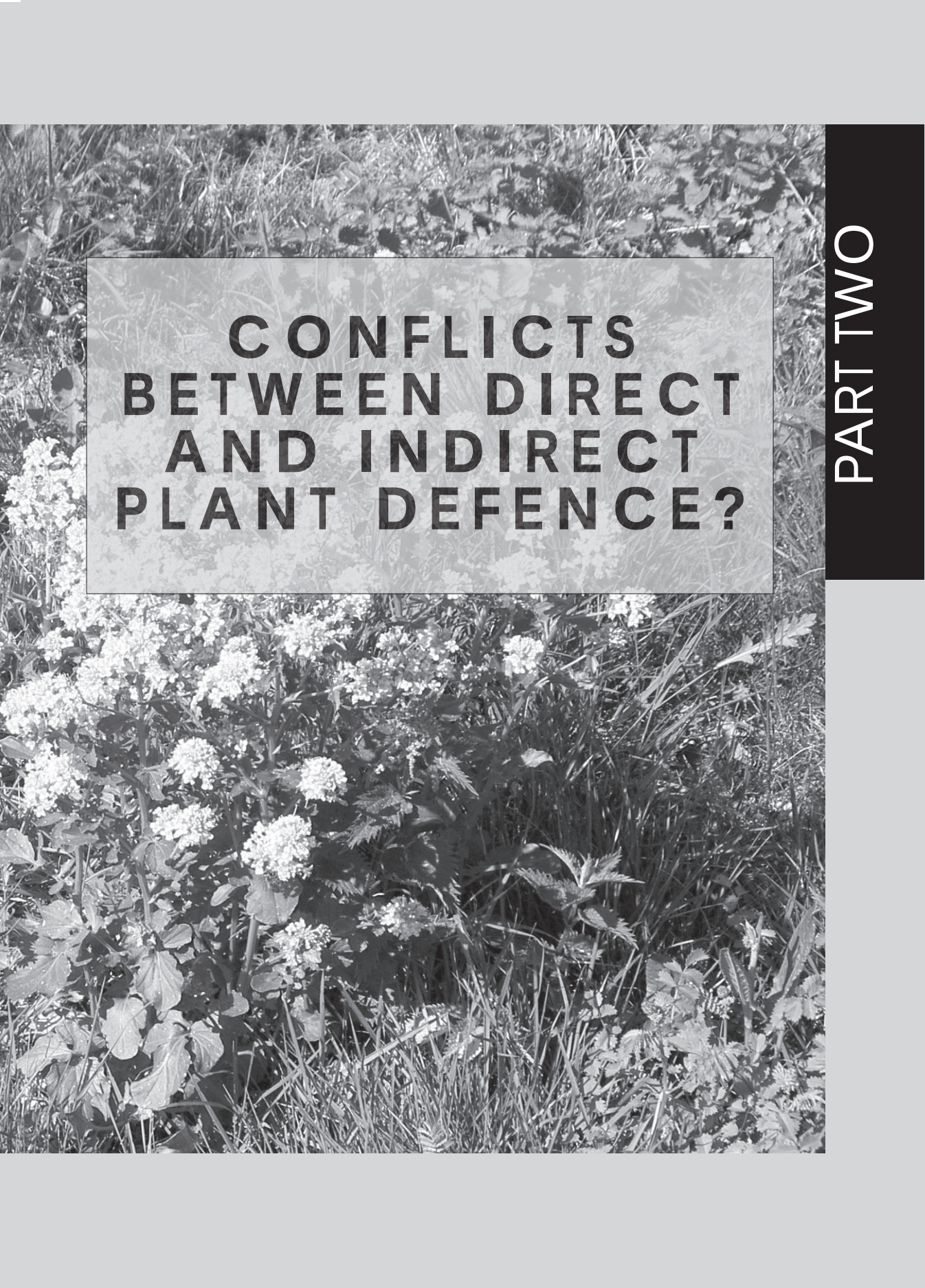
Because we studied interactions involving only single pairs of generalist and specialist herbivores and their endoparasitoids, these results cannot be used to predict differential herbivore

pressures in natural populations of *B. oleracea*. It is known that herbivory may be a major factor in driving the evolution of direct plant defences over comparatively large spatial scales (Zangerl and Berenbaum 2005). However, little is currently known about the factors that maintain the dramatic differences in secondary chemistry of the wild *B. oleracea* populations. Studies are planned in which the population dynamics of both herbivores and parasitoids will be examined in different wild populations of *B. oleracea* growing along the British coastline. These studies are aimed at elucidating a suite of biotic and abiotic factors that mediate the variation in secondary plant chemistry in *B. oleracea*.

Acknowledgements

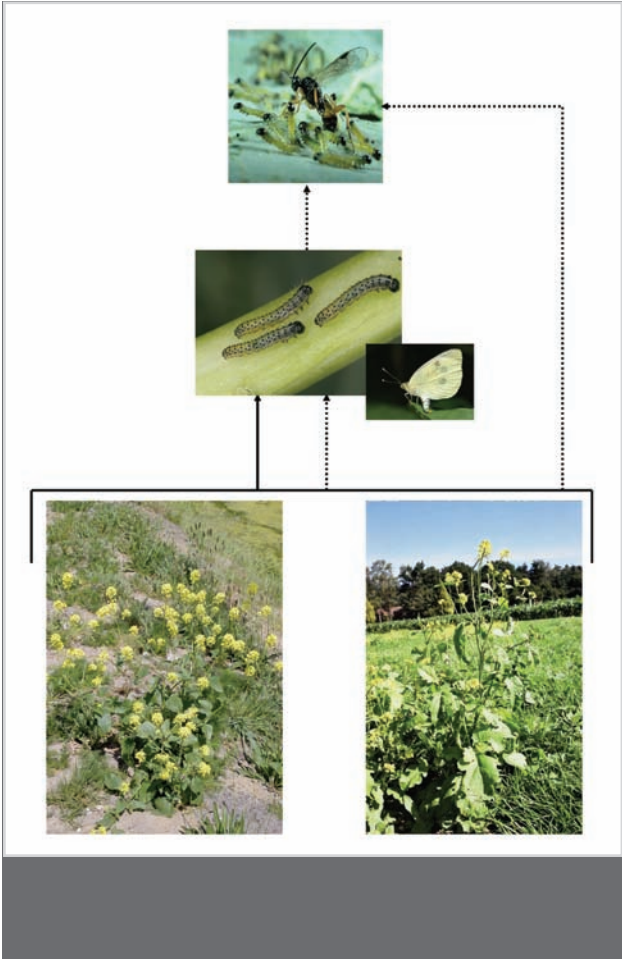
We would like to thank Leo Koopman and André Gidding for rearing of insects and Ciska Raaijmakers for assistance with the glucosinolate analyses.



A black and white photograph of a field. In the foreground, there are several tall, leafy plants with clusters of small white flowers. The background is filled with a dense field of similar plants and tall grass. A semi-transparent rectangular box is overlaid on the middle of the image, containing the title text.

CONFLICTS BETWEEN DIRECT AND INDIRECT PLANT DEFENCE?

PART TWO



6

The effect of direct and indirect defences in two wild brassicaceous plant species on a specialist herbivore and its gregarious endoparasitoid.

by R. Gols, L.M.A. Witjes, J.J.A. van Loon, M.A. Posthumus,
M. Dicke & J.A. Harvey

To be published in *Entomologia Experimentalis et Applicata* (in press)

Most studies on plant defences against insect herbivores investigate direct and indirect plant defences independently. However, these defences are not necessarily mutually exclusive. Plant metabolites can be transmitted through the food chain and can also affect the herbivore's natural enemies. A conflict may arise when a natural enemy is attracted to a plant that is suboptimal in terms of its own fitness. In addition, plant defences are often studied in cultivated plant species in which artificial selection may have resulted in reduced resistance against insect herbivores. In this study we investigated both direct and indirect plant defences in two closely related wild brassicaceous plant species, *Brassica nigra* L. and *Sinapis arvensis* L. The herbivore *Pieris brassicae* L. (Lepidoptera: Pieridae), which is specialised on brassicaceous plant species, developed faster and attained higher pupal mass when reared on *B. nigra* than on *S. arvensis*. In contrast, *Cotesia glomerata* L. (Hymenoptera: Braconidae), which is a gregarious endoparasitoid of *P. brassicae* caterpillars, developed equally well on *P. brassicae* irrespective of the food plant on which its host had been reared. The feeding strategy of the parasitoid larvae, i.e., selectively feeding on haemolymph and fat body, is likely to allow for a much wider host-size range without affecting the size or development time of the emerging parasitoids. In flight chamber experiments, *C. glomerata*, which had an oviposition experience in a host that fed on Brussels sprout, exhibited significant preference for host-damaged *B. nigra* over host-damaged *S. arvensis* plants. Headspace analysis revealed quantitative and qualitative differences in volatile emissions between the two plant species. This parasitoid species may use a range of cues associated with the host and the host's food plant in order to recognise the different plant species on which the host can feed. These results show that there is no conflict between direct and indirect plant defences for this plant-host-parasitoid complex.

Key words: *Brassica nigra*, *Cotesia glomerata*, crucifers, development, glucosinolates, herbivore-induced plant volatiles, *Pieris brassicae*, *Sinapis arvensis*, Lepidoptera, Pieridae, Hymenoptera, Braconidae

Introduction

Plants have evolved several defence traits to prevent or reduce feeding by insect herbivores. These traits can be broadly divided into direct and indirect defences. Direct plant defences refer to plant traits that negatively affect development of the herbivore, whereas indirect defences refer to traits that promote the effectiveness of the herbivore's natural enemies. Examples of direct defences are morphological characteristics such as spines and wax layers, which may hamper colonisation and movement on the plant (Schoonhoven et al. 2005). Furthermore, the production of chemicals such as toxins and digestibility reducers may interfere with the physiology of the herbivore and reduce growth and survival (Schoonhoven et al. 2005). Indirect plant defences include the provisioning of alternative food sources and shelter for the herbivore's natural enemies, and the production of volatile attractants. It has been demonstrated for many plant species that herbivore damage induces the release of volatile chemicals that attract parasitoids and predators. These herbivore-induced plant volatiles can be used by the natural enemies of herbivores to locate plants infested with their prey or hosts (Dicke 1999a; Paré et al. 1999; Sabelis et al. 1999; Vet 1999; Turlings et al. 2002).

A conflict between direct and indirect plant defences may arise when plants have high levels of direct defence compounds and at the same time are also highly attractive to the insect's natural enemies (Havill and Raffa 2000). It has been demonstrated that plant secondary chemicals such as toxins not only affect development of insect herbivores but also that of higher trophic levels (reviewed by Harvey 2005; Ode 2006). As direct and indirect plant defences are usually studied independently, the occurrence of conflicts between different defensive traits is rarely addressed. Furthermore, direct and indirect plant responses do not act independently of each other. For instance, the plant hormone jasmonic acid plays an important role in the induction of direct as well as indirect plant defences (reviewed by Dicke and van Poecke 2002). In order to better understand the evolution of plant defences against arthropod herbivores, plant defences should also be studied in wild plant species.

Research exploring evolutionary aspects of interactions between plants, herbivores, and their natural enemies in a multitrophic framework has often employed crop plants in stead of their wild relatives. Plant breeding programs may have disrupted the original plant defence strategies that were present in wild progenitors of cultivated plants that evolved under natural selection (Evans 1993; Rosenthal and Dirzo 1997). Artificial selection of crop plants has in some cases reduced the level of secondary plant compounds and may have altered the strength of indirect plant responses as well. Therefore, it is even more important to compare the chemistry of crop plants with their wild

relatives and to determine how this affects the behaviour and development of associated insect consumers (van der Meijden and Klinkhamer 2000).

In this study, we investigated both direct and indirect plant defences in two closely related wild plant species, *Brassica nigra* L. and *Sinapis arvensis* L. (Brassicaceae). Both species are native to much of Eurasia, including The Netherlands, and belong to the large family Brassicaceae, which is characterised by the production of glucosinolates (GS). Hydrolyzed metabolites of GS, which are produced in response to tissue damage, play an important role in defence of Brassicaceae against insect herbivory (Chew 1988; Rask et al. 2000). Some insect species have evolved mechanisms to detoxify GS (Ratzka et al. 2002; Wittstock et al. 2004). Insects that are specialist feeders on plants containing GS may even use these chemicals as feeding stimulants (van Loon et al. 1992; Bartlett et al. 1994; Renwick 2002) or sequester them and employ them for their own defence (Müller et al. 2001; Aliabadi et al. 2002). However, high levels of GS breakdown products can also have a negative effect on the development of specialist herbivores (Agrawal and Kurashige 2003). Furthermore, different brassicaceous plant species not only vary in food plant quality for hosts specialised on plants in this family but also for their endoparasitoids (Ohsaki and Sato 1994; Harvey et al. 2003; Sznajder and Harvey 2003; Harvey and Wagenaar 2006).

Pieris brassicae L. (Lepidoptera: Pieridae) is a specialist herbivore that feeds exclusively on plants producing GS. Larvae are often considered to be pests of cultivated crucifer species (e.g., *Brassica oleracea*), but also readily feed on related wild species in the Brassicaceae such as *B. nigra* and *S. arvensis* (Feltwell 1982). *Cotesia glomerata* L. (Hymenoptera: Braconidae) is a fairly specialised gregarious endoparasitoid that primarily attacks first to third instars of *P. brassicae* and a related species, *P. rapae*, in Eurasia (where all three species are native). Larvae of *C. glomerata* feed primarily on their host's haemolymph and fat body before emerging through the side of the host to pupate.

The aims of this study were (1) to investigate whether *B. nigra* and *S. arvensis* differ in plant quality for *P. brassicae* as well as for its larval endoparasitoid, *C. glomerata*, and (2) to determine whether female *C. glomerata* wasps are differentially attracted to the two plant species when damaged by *P. brassicae* larvae. In addition, we measured GS concentrations in leaf tissue and described the chemical composition of the herbivore-induced volatile blend emitted by the two plant species. Differences and similarities in herbivore and parasitoid performance, respectively, are discussed in relation to GS content and parasitoid feeding biology. Furthermore we discuss the dynamic response of *C. glomerata* to different host-plant species that emit quantitatively and qualitatively different volatiles blends.

Materials and methods

Plants

Seeds for *B. nigra* and *S. arvensis* were obtained from large, single populations growing within 2-10 km of Wageningen The Netherlands (coordinates *B. nigra* 51°58'N, 5°38'E and *S. arvensis* 51°57'N, 5°48'E). Plants of both species were grown in pots (20 × 25 cm) containing a soil mixture consisting of approximately 30% sand, 5% clay, and 65% peat. Plants were reared in a climate-controlled greenhouse at $25 \pm 2^\circ\text{C}$, 50% r.h., and a photoperiod of at least 16 h. If the light dropped below 500 $\mu\text{mol photons/m}^2/\text{s}$ during the 16-h photoperiod, supplementary illumination was applied by high-pressure mercury lamps. Plants were watered daily. In the experiments we used non-flowering plants that were approximately 4 weeks old.

Insects

The herbivore, *P. brassicae*, and its larval endoparasitoid, *C. glomerata*, were originally collected from Brussels sprout (*Brassica oleracea* var. *gemmifera*) fields in the vicinity of WU. Cultures of *P. brassicae* and *C. glomerata* were reared on Brussels sprout (cv. Cyrus) in climate controlled rooms at $22 \pm 2^\circ\text{C}$, 50% r.h., and a L16:D8 photoperiod. The parasitoid, *C. glomerata*, was reared from *P. brassicae*. A leaf infested with first (L1) or second (L2) instars was placed in the rearing cage with adult wasps for 10-30 min, depending on the density of the wasps. The leaf with parasitised larvae was then transferred to a cage containing cabbage plants. After approximately 2 weeks, when the host caterpillars were late L5 stage, the parasitoid larvae egressed in preparation for pupation. Parasitoid cocoons were collected and transferred to a new cage until emergence of the adult wasps. The wasps were supplied with 10% (wt/vol) sucrose dissolved in tap water. Wasps were used either for rearing or experimental purposes.

Plant quality for development of the herbivore and its parasitoid

Neonate larvae of *P. brassicae* were placed in groups on leaves of the two crucifer species in cages (40 × 70 × 50 cm) and kept in climate room facilities at $25^\circ \pm 2^\circ\text{C}$, 50% r.h., and a L16:D8 photoperiod. Approximately 30 caterpillars were kept in each cage to prevent overcrowding and to standardise rearing conditions. First instars were allowed to feed and develop on their respective food plants until the 2nd day. At this stage, for each food plant, one cohort of 54 larvae was parasitised by *C. glomerata*, whereas a second cohort of 32 larvae served as unparasitised control. Caterpillars of *P. brassicae* were individually presented to parasitoid females in plastic vials. Wasps were allowed to

parasitise the host once. The completion of the oviposition of a single brood by *C. glomerata* normally takes more than 10 s; hosts from which females removed their ovipositor within 5 s were assumed to be rejected. None of the hosts offered to the wasps was rejected. More than 20 female wasps were used to parasitise 108 larvae.

Following parasitism, hosts were returned to their respective food plants in rearing cages with a maximum of four plants in a single cage. Unparasitised larvae were kept in separate cages on their respective food plant. Plants were added every 3 days or earlier if insufficient leaf material remained for feeding. Approximately 24 h prior to larval parasitoid egression and pupation, host caterpillars entered the wandering phase and left the food plants by climbing to the top of the cage. From here they were removed from the cages and maintained in labelled 9.5-cm Petri dishes until parasitoid egression and pupation. The number of parasitoid larvae that egressed from a single host (= secondary brood size) was recorded. The still-living host was separated from the cocoons and weighed on a Sartorius AG, Göttingen, Germany microbalance (1 μ g accuracy).

The remaining parasitoid cocoons were checked several times daily for adult parasitoid emergence. Unparasitised larvae also leave the plant when larval development is completed and pupate on the cage walls. For each pupa the day of pupation and their fresh weight was recorded. Upon eclosion adult *C. glomerata* wasps were killed by freezing. They were then segregated by sex and brood (i.e., wasps emerged from a single caterpillar) and weighed. The mean body masses of male and female wasps per brood were obtained by dividing the total mass of emerged wasps by the number of wasps in the brood. In order to ensure that fresh mass is an efficient index of parasitoid size, a sub-sample of male and female wasps from five broods originating from each plant (= 10 in total) were dried in an oven at 60 °C for 48 h, and then weighed on the microbalance. Development time of male and female parasitoids was recorded in days. Most parasitoids from a single brood emerged between 08:00 and 15:00 hours on the same day; however, wasps that emerged after 21:00 h were assumed to emerge on the following day. Development time was determined as the number of days between parasitism and adult emergence. Offspring sex ratios were determined only for *C. glomerata* that produced mixed broods ($n = 47$ in *B. nigra* and $n = 43$ in *S. arvensis*).

Glucosinolate analyses

Samples for GS analyses were taken during the host-plant-quality experiment from both *S. arvensis* ($n = 9$) and *B. nigra* plants ($n = 8$). After the plants had been damaged by *P. brassicae* for 3 days, all remaining leaf tissue was collected. Immediately after harvesting, samples were frozen at -20 °C. Fifty mg aliquots of

freeze-dried and pulverised material were used for GS analysis. GS were extracted with boiling 70% methanol, desulfated and separated by means of HPLC (for a detailed description of the method see van Dam et al. 2004).

Flight response of *Cotesia glomerata* to *Brassica nigra* and *Sinapis arvensis* plants infested with *Pieris brassicae*

The flight response of *C. glomerata* was tested in a greenhouse compartment at $22 \pm 2^\circ\text{C}$ and 60% r.h. in a tent-like structure ($330 \times 175 \times 280$ cm), which was made of white nylon sheets. The greenhouse compartment was continuously ventilated, which created a turbulent air-current inside the tent. In the bioassay we placed two plants, each of a different treatment (see below), on a table 60 cm apart. *Cotesia glomerata* females were released in vials at one end of the table 100 cm away from the plants. Female wasps were collected from the mass rearing before using them in the bioassay by putting a leaf of a Brussels sprout plant with L1 or L2 *P. brassicae* larvae in the rearing cage for less than a min. Females were allowed to oviposit and were collected from this leaf with a fine paintbrush and transferred to 5 ml-vials (one wasp per vial). Oviposition experience enhances the motivation of the wasps to forage for hosts. As wasps do not leave the vials instantaneously, about 10 vials were placed on the table simultaneously. Wasps were allowed to fly freely within the tent. As soon as a wasp landed on one of the two plant species, it was collected and the species on which it had landed was recorded. If some of the wasps that were initially released had not alighted on one of the plants within 30 min, additional wasps were released. The position of the two plants was exchanged after five wasps had landed.

Experiment 1: undamaged vs., damaged plants. In this experiment, we tested the response of female wasps to non-infested clean plants vs. plants damaged by 20 L1 *P. brassicae* larvae for 96 h for both plant species separately. For each plant species, we conducted three replicates on different experimental days with 10-15 wasps in each replicate. Wasps were only used once.

Experiment 2: damaged Brassica nigra vs. damaged Sinapis arvensis. In this experiment, we tested the two plant species against each other. Plants had been infested with 20 L1 larvae of *P. brassicae* and were tested in the flight chamber 1, 2, 3, and 4 days after the caterpillars had been placed on the plants. Plants had been labelled and the two plants were tested again on three successive days in the same combination. Wasps were not allowed to search for hosts and oviposit on *B. nigra* and *S. arvensis*. Each wasp was only used once on an experimental

day, after which it was returned to the culture. A total of 10 wasps were recorded per set of plants per day. In total we used 18 sets of plants, which were tested in two blocks of four experimental days with approximately 400 different females.

Headspace collection and analyses

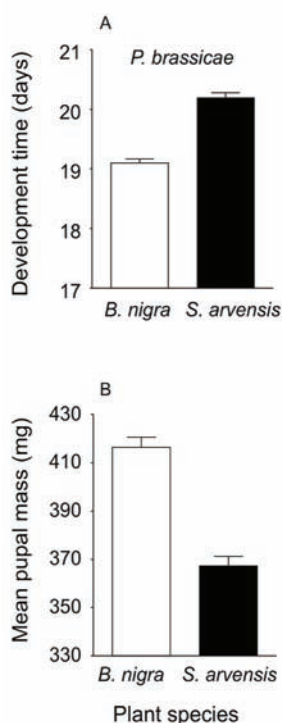
Plants were infested with 20 L1 larvae of *P. brassicae*, as in the flight chamber bioassay. Larvae were allowed to feed for 4 days before the plants (with the caterpillars) were transferred to 35-l glass collection containers. Pressurised air was filtered over silica gel, molecular sieves 4Å (Sigma, St Louis MO, USA) and activated charcoal before entering the sampling container. Prior to sampling, the container was purged for 1 h. Subsequently, the headspace volatiles were trapped on Tenax TA (90 mg, 20/35 mesh; Chrompack, Middelburg, The Netherlands) for 4 h at 100 ml/min. Headspace collection was conducted in five replicated experiments for both plant species in a climate controlled room $22^{\circ} \pm 1^{\circ} \text{C}$ with mercury lamps ($40 \pm 10 \mu\text{mol photons/m}^2/\text{s}$) hanging above the pots.

The collected volatiles were released from the Tenax by heating in a Thermodesorption Cold Trap Unit (Chrompack) at 250°C for 10 min and flushing with a helium flow of 10 ml/min. The released compounds were cryofocused in a coldtrap (0.52 mm i.d. deactivated fused silica) at a temperature of -85°C . The volatiles were transferred to the GC-column (Supelcowax 10 fused silica capillary column; $60 \text{ m} \times 0.25 \text{ mm i.d.}$, $0.25 \mu\text{m}$ film thickness) by ballistic heating of the cold trap to 220°C . The GC-column was connected to a Finnigan MAT 95 mass spectrometer, Bremen, Germany and programmed from 40°C (3 min hold) to 270°C (4 min hold) at $4^{\circ}\text{C}/\text{min}$ and the initial helium flow rate was 30 cm/s. The mass spectrometer was operated in the 70 eV EI ionisation mode and scanning was done from mass 24 to 400 at 0.7 s/decade. Compounds were identified by comparison of the mass spectra with those in the Wiley library and in the Wageningen Mass Spectral Database of Natural Products and by checking the retention index.

Statistical analyses

Two-sample t-tests were used to compare pupal mass and development time of *P. brassicae* on the two plant species. We also used t-tests to reveal difference in brood size and sex ratio (number of males divided by the total number of wasps) of the parasitoid on the two plants species. Parasitoid development time and body mass were analysed by ANOVA with food plant (*B. nigra*, *S. arvensis*), offspring sex (male, female), and their interaction as explanatory variables. Post-hoc multiple comparisons were made using Tukey-Kramer tests. We used plant species as treatment and brood size as a covariate to determine the relationship

Figure 1: Development of *Pieris brassicae* reared on *Brassica nigra* (white bars) and *Sinapis arvensis* (black bars) plants; (A) Larva-to-pupa development time (mean + SE days) and (B) pupal fresh mass (mean + SE mg).



between fresh mass of the host carcass after emergence of the parasitoid larvae and brood size on the two plant species. The relationship between fresh and dry mass of the adult parasitoids was determined using linear regression analyses.

For the volatile data, we used two-sample t-tests for each of the compounds to reveal differences in emissions by the two plant species. Peak areas were log-transformed to meet assumptions of normality. To test whether the duration of feeding by *P. brassicae* had an effect on the landing preference of *C. glomerata*, we used a generalised linear model with binomial distribution for errors and a logit link function. The response variable was the fraction out of 10 wasps that chose *B. nigra* over *S. arvensis*. Duration of feeding (1, 2, 3, and 4 days) and trial (18 sets of plants) were entered as explanatory variables in the regression model. To determine whether *C. glomerata* landing responses were significantly different from a no-preference situation, we conducted two tailed Z-tests on arcsine transformed ratios ($H_0: \mu = \arcsin 0.5$). These Z-tests were performed separately for each of the time points (i.e., after 1, 2, 3, and 4 days of feeding by *P. brassicae*). Analysis was carried out using SAS 8.2. (SAS Institute Inc., Cary NC, USA).

Results

Plant quality for development of the herbivore and its parasitoid

The success of *P. brassicae* development, based on survival from newly hatched L1 larvae to adulthood, did not differ significantly between the two food-plant species. Not a single caterpillar failed to pupate, and only one pupa (out of 32) reared on *S. arvensis* died prior to adult emergence. In contrast, there was a significant effect of food plant species on the duration of development between larval hatching and pupation ($t_{59} = 12.8$, $P < 0.001$; Fig. 1A). *Pieris brassicae* took just over 24 h longer to pupate when reared on *S. arvensis* compared with *B. nigra*. Furthermore, pupal weight differed significantly with plant species on which *P. brassicae* was reared ($t_{56} = 8.71$, $P < 0.001$; Fig. 1B). Individuals were, on average, about 50 mg larger when developing on *B. nigra* than on *S. arvensis*.

Among parasitised larvae of *P. brassicae*, the mass of the host carcass following larval parasitoid emergence was different for the two plant species ($F_{1,104} = 17.9$, $P < 0.001$) and covaried with secondary brood size in *C. glomerata* (with equal slopes $F_{1,104} = 20.0$, $P < 0.001$). The statistical model including the size-plant interaction term, which tests for unequal slopes, was not significant; therefore, the interaction term was omitted from the model. For a given brood size, caterpillars reared on *B. nigra*

were approximately 25 mg larger than conspecific larvae reared on *S. arvensis* (Fig. 2).

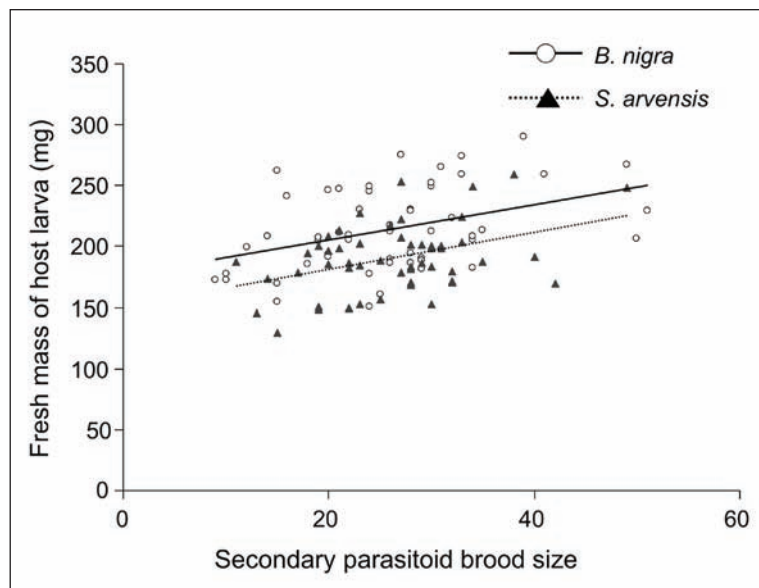


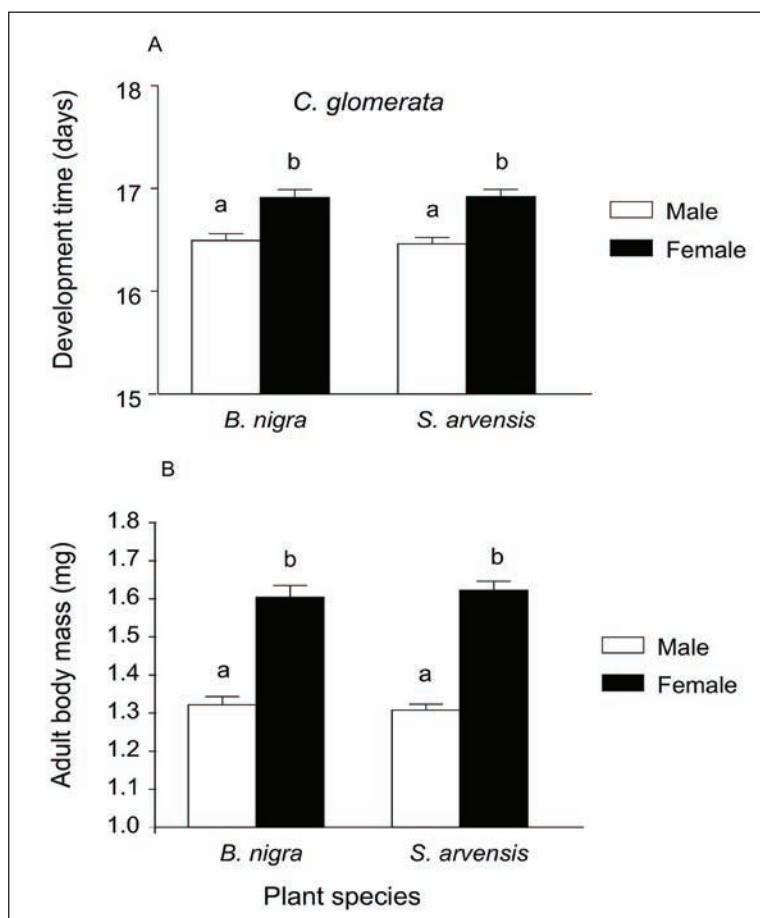
Figure 2: Relation between fresh mass of *Pieris brassicae* caterpillars after emergence of the parasitoid *Cotesia glomerata* and secondary brood size when the host was reared on *Brassica nigra* (open circles) or *Sinapis arvensis* plants (closed triangles).

Secondary brood size in *C. glomerata* did not vary significantly with the food plant on which the host had been reared ($t_{105} = 0.14$, $P = 0.89$). The brood size of *C. glomerata* was 26.1 ± 1.3 (mean $\pm$ SE) on *B. nigra* and 26.3 ± 1.0 on *S. arvensis*. Remarkably, 100% (54 of 54) and 98% (53 of 54) of parasitised *P. brassicae* larvae on *B. nigra* and *S. arvensis*, respectively, produced parasitoid broods. Also, offspring sex ratio did not differ ($t_{88} = 0.60$, $P = 0.55$) in broods reared on *B. nigra* (% of males per brood, mean $\pm$ SE: 53.0 ± 4.2) and *S. arvensis* (49.6 ± 3.9). Development time in *C. glomerata* (Fig. 3A) varied significantly with offspring sex ($F_{1,193} = 40.4$, $P < 0.001$) but not between the two crucifer plant species ($F_{1,193} = 0.04$, $P = 0.84$), nor was the interactive effect of these parameters on development time significant ($F_{1,193} = 0.07$, $P = 0.78$). *Cotesia glomerata* is protandrous, with male wasps emerging about 12 h earlier than females. Similarly, adult parasitoid mass (Fig. 3B) varied significantly with offspring sex ($F_{1,193} = 168$, $P < 0.001$), but not with food plant species ($F_{1,193} = 0.01$, $P = 0.92$) and the interactive effect of these parameters also did not significantly affect body mass ($F_{1,193} = 0.47$, $P = 0.50$). Female wasps were typically some 20% larger than their male counterparts (Fig. 3B). As fresh body mass correlated significantly with dry mass in both males ($F_{1,9} = 90.2$, $P < 0.001$, $r^2 = 0.92$) and females ($F_{1,9} = 90.2$, $P < 0.001$, $r^2 = 0.97$), size data were based on measurements of fresh mass.

Glucosinolate analyses

GS analysis of leaf tissue revealed qualitative and quantitative differences between the two plant species. In *S. arvensis* plants, sinalbin was the only detectable GS and reached levels of 7.6 ± 2.9 (mean $\pm$ SE) $\mu\text{mol/g}$ dry weight (DW) ($n = 9$ plants). In *B. nigra*, sinigrin was the major GS (>98% of total GS content) and was present in levels of 16.9 ± 1.9 $\mu\text{mol/g}$ DW ($n = 8$ plants). Other GS detected in *B. nigra* were glucobrassicin (0.045 ± 0.006 $\mu\text{mol/g}$ DW), neoglucobrassicin (0.003 ± 0.001 $\mu\text{mol/g}$ DW), 4-methoxy glucobrassicin (0.05 ± 0.02 $\mu\text{mol/g}$ DW) and 4-hydroxy glucobrassicin (0.037 ± 0.006 $\mu\text{mol/g}$ DW).

Figure 3: Development of *Cotesia glomerata* from *Pieris brassicae* reared on *Brassica nigra* or *Sinapis arvensis* plants for males (white) and females (black); (A) egg-to-adult development time (mean $\pm$ SE days) and (B) adult fresh mass (mean $\pm$ SE mg). Bars with the same letter are not significantly different (Tukey-Kramer multiple comparisons, $P < 0.05$).



Flight response of *Cotesia glomerata* to *Brassica nigra* and *Sinapis arvensis* plants infested with *Pieris brassicae*

Cotesia glomerata females, which had an oviposition experience in individual hosts feeding on Brussels sprout, clearly preferred

host-damaged plants over clean undamaged plants. In *S. arvensis*, 93% (28 out of 30) of the wasps landed on herbivore-damaged plants, whereas in *B. nigra* 95% (37 out of 39) of the wasps preferred damaged plants over undamaged plants in a two-choice situation. When host-damaged plants of the two species were offered together, *C. glomerata* had a significant preference to land on *B. nigra* plants over *S. arvensis* at day 3 and day 4 since larval feeding was initiated (Fig. 4). Feeding duration had no effect on the preference of the wasps ($\chi^2 = 0.21$, d.f. = 3, $P = 0.98$). The effect of trial bordered significance ($\chi^2 = 26.8$, d.f. = 17, $P = 0.06$). The effect of duration of induction was not influenced by an interaction with trial ($\chi^2 = 60.3$, d.f. = 51, $P = 0.17$).

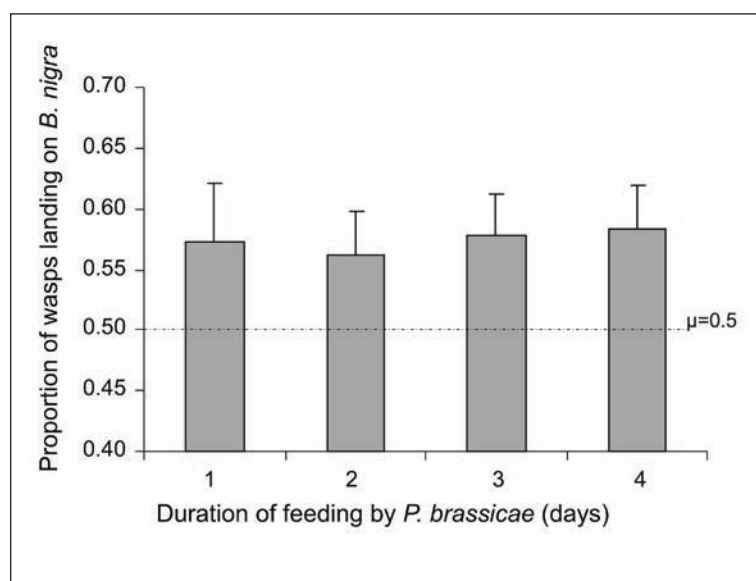


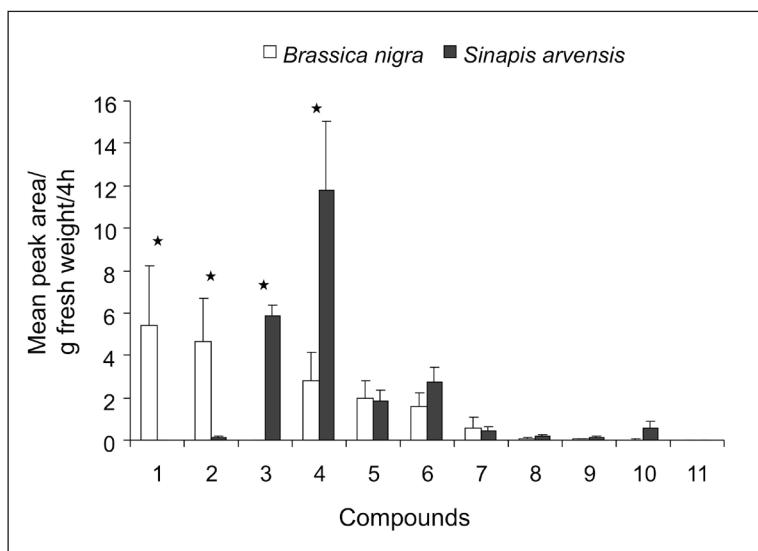
Figure 4: Landing preference of female *Cotesia glomerata* when given the choice between *Brassica nigra* and *Sinapis arvensis* plants that had been damaged by 20 first instar *Pieris brassicae* larvae for 1, 2, 3, and 4 days. Each bar represents the mean ($\pm$ SE; $n = 18$ trials) proportion of wasps landing on *B. nigra* out of a total of 10 wasps. Statistical analysis revealed that preference for *B. nigra* plants was not significant after 1 ($t_{17} = 1.67$, $P = 0.11$) and 2 days ($t_{17} = 1.88$, $P = 0.08$) of feeding by *P. brassicae*, and significant after 3 ($t_{17} = 2.22$, $P = 0.04$), and 4 days ($t_{17} = 2.37$, $P = 0.03$) of feeding.

Headspace analyses

In the headspace of both *B. nigra* and *S. arvensis* that had been fed upon by *P. brassicae* larvae for 4 days, 11 different compounds were detected, of which nine were present in both plant species (Fig. 5). In *S. arvensis*, the major compound was (*Z*)-3-hexen-1-yl-acetate, which formed 48% of the total volatile blend, followed by β -caryophyllene, which contributed 25% to the total blend. (*Z*)-3-hexen-1-yl-acetate was emitted at significantly lower rates by *B. nigra*, whereas β -caryophyllene was not produced by this plant species. Allyl isothiocyanate together with (3*E*)-4,8-dimethyl-1,3,7-nonatriene (= DMNT) were the most dominant compounds in the headspace of *B. nigra* plants, constituting 41 and 33% of the total blend, respectively. These two compounds were either absent (allyl isothiocyanate) or produced in only small amounts (DMNT) by *S. arvensis* plants. Superficial inspection of the plants

after 4 days of feeding did not reveal any difference in the amount of tissue that had been consumed from the two plant species.

Figure 5: Composition of the volatile blend emitted by *Brassica nigra* (white bars) and *Sinapis arvensis* (black bars) plants after 96 h of feeding damage by *Pieris brassicae*. At $t = 0$, 20 first instars were placed on the plants, which were left on the plant during sampling. Each bar depicts the mean peak area/g fresh weight of above-ground tissue ($\pm$ SE; $n = 5$). Compounds: (1) allyl isothiocyanate, (2) (3E)-4,8-dimethyl-1,3,7-nonatriene (= DMNT), (3) β -caryophyllene, (4) (Z)-3-hexen-1-yl acetate, (5) (Z)-3-hexen-1-ol, (6) 2-ethyl-1-hexanol, (7) benzothiazole, (8) unknown compound, (9) 3-heptanone, (10) 2-heptanone, and (11) dimethyl trisulfide. Bars with an asterisk denote means that differ significantly between the two plant species.



Discussion

The results of this investigation reveal that some fitness-related parameters (development time and body mass) of the insect herbivore, *P. brassicae*, differed when reared on *B. nigra* or *S. arvensis*. *Pieris brassicae* developed faster and pupae were larger when reared on *B. nigra* than on *S. arvensis*, although survival (to adult) was unaffected. In contrast, the identity of the plant species had little effect on the fitness-related traits of the parasitoid, *C. glomerata*. Although male wasps were smaller and developed faster than female conspecifics, there was little difference in quality between *B. nigra* and *S. arvensis*. Several studies have reported that development of herbivores and/or their parasitoids varies when feeding on different plant species or genotypes (Ohsaki and Sato 1994; Benrey et al. 1998; Harvey et al. 2003, 2005, 2007a; Ode et al. 2004; Gols et al. 2008c;). This indicates that plant quality varies due to differences in such factors as primary and/or secondary metabolites.

Hydrolysis products of GS, which are produced after cell rupture, have been shown to play a defensive role against insect herbivores (Chew 1988; Rask et al. 2000). GS analyses of leaf tissues revealed that *B. nigra* contains mainly sinigrin (allyl GS) whereas *S. arvensis* contains only sinalbin. *Pieris brassicae* has evolved an efficient detoxification mechanism, which facilitates the conversion of GS into nitriles, instead of the much more toxic isothiocyanates (Wittstock et al. 2004). However, Agrawal

& Kurashige (2003) reported that even highly specialised insect herbivores, such as *P. rapae* (closely related to *P. brassicae*) can be negatively affected by GS such as sinigrin. A more recent study demonstrated that growth of *P. brassicae* is not negatively influenced by relatively high levels of sinigrin present in *B. nigra* leaf tissue (Smallegange et al. 2007). Moreover, when reaching the second and third instar, many *P. brassicae* caterpillars move to the flowers, which contain significantly higher amounts of sinigrin than leaves, and the larvae attain higher biomass when they selectively feed on flowers (Smallegange et al. 2007). For sinalbin, it has been shown that *P. brassicae* prevents the formation of toxic hydrolysis products by converting sinalbin (4-hydroxybenzyl GS) into 4-hydroxybenzylcyanide sulfate, with the nitrile 4-hydroxybenzylcyanide as intermediate (Agerbirk et al. 2006). Likewise, sinigrin in *B. nigra* may also be converted into less toxic metabolites. Consequently, *P. brassicae* is very efficient in detoxifying sinigrin and sinalbin, and therefore it is unlikely that these GS have a large impact on the performance of *P. brassicae*.

Morphologically, *B. nigra* and *S. arvensis* are quite similar, although *B. nigra* leaves have higher densities of trichomes than *S. arvensis* leaves. As caterpillars performed better on *B. nigra* plants, hairiness did not apparently hamper the movement and feeding behaviour of *P. brassicae* caterpillars, or else any negative effects were cancelled out by other positive plant characteristics. The two plant species may have differed in levels of other defence-related compounds such as digestibility reducers. In addition, concentrations of primary metabolites (e.g., amino acids), which are also important factors determining food plant quality for insect herbivores (Slansky 1993), may have differed between the two plant species.

Although host development was significantly affected by food plant species, parasitoid performance was not. Most importantly, for a given parasitoid brood size, the remaining mass of the host carcass after egression of the parasitoid larvae was significantly smaller in hosts reared on *S. arvensis* than in hosts reared on *B. nigra*. Larvae of *C. glomerata*, like their close relatives in the braconid subfamily Microgastrinae, feed primarily on host haemolymph and fat body during their development and thus consume only a fraction of available host resources (Harvey 2005). This suggests that *C. glomerata* larvae are able to adjust their feeding behaviour in accordance with differences in host size, and thus consumed a higher proportion of the mass of smaller hosts reared on *S. arvensis* than larger hosts reared on *B. nigra*.

In the flight chamber experiments, *C. glomerata* had a significant preference for *B. nigra* over *S. arvensis* plants and this preference remained constant irrespective of the duration of feeding by *P. brassicae*. Headspace analyses revealed significant

quantitative and qualitative differences between the two plant species. Herbivore-damaged *B. nigra* plants produced high levels of allyl isothiocyanate and the terpenoid DMNT, whereas *S. arvensis* produced the sesquiterpenoid β -caryophyllene and significantly higher amounts of the green leaf volatile (Z)-3-hexen-yl-acetate. In a time course experiment, Scascighini et al. (2005) found that *C. glomerata* was able to discriminate between infested and uninfested cabbage plants after only 1 h of feeding by *P. brassicae*. The response of the wasp reached a maximum after 3 h of feeding and remained constant for the subsequent 17 h (Scascighini et al. 2005). Our results suggest that the difference in attractiveness between *B. nigra* and *S. arvensis* remains constant for at least 4 days.

Although *C. glomerata* females preferred *B. nigra* plants, many (ca. 45%) still initially alighted on *S. arvensis*. In addition, *C. glomerata* females discriminated between undamaged and host-damaged *S. arvensis* plants. Therefore, the headspace of *S. arvensis* was also highly attractive to this parasitoid. Before the wasps were released in the flight bioassay, they had an oviposition experience in a host feeding on Brussels sprout. Previous work has demonstrated that the volatile blend emitted by Brussels sprout is much more complex (>70 compounds, see Mattiacci et al. 1994) than volatile blends emitted by *B. nigra* and *S. arvensis*. However, we cannot exclude the possibility that previous oviposition experience in larvae feeding on Brussels sprout has enhanced the preference for *B. nigra* over *S. arvensis* through associative learning (Vet et al. 1995).

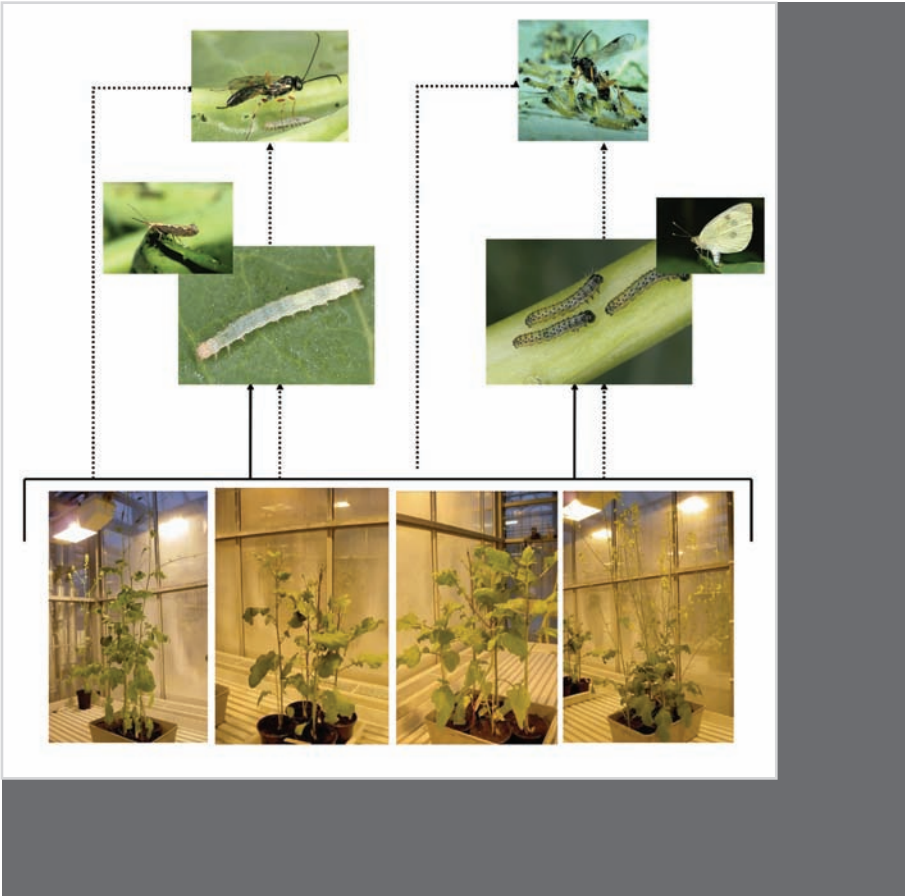
Our results also suggest that the response of *C. glomerata* to herbivore-induced plant volatiles is dynamic. This parasitoid species may use a range of cues in order to recognise the different plant species that are food plants of the host. Alternatively, the parasitoid may use general plant signals to locate host-food plants from a distance, whereas cues from the host itself may play a role in host recognition once the food plant of the host is found. Frass produced by *P. rapae* larvae that had been feeding on cabbage plants emits isothiocyanates, which are breakdown products of GS (Agelopoulos and Keller 1994a; Agelopoulos et al. 1995). In addition *C. glomerata* discriminates between undamaged plants with frass from *P. brassicae* and undamaged plants without frass (Steinberg et al. 1993). If breakdown products of GS play a role in host plant selection of *C. glomerata*, high amounts of allyl isothiocyanate occurring in *B. nigra* plants may explain the enhanced attraction of this plant species to *C. glomerata*. For example, *Diaeretiella rapae*, which is a parasitoid of aphids mainly feeding on *Brassica* plant species, is attracted to synthetic 3-butenyl isothiocyanate (Blande et al. 2007), as well as to *B. napus* plants containing high levels 3-butenyl isothiocyanate (Bradburne and Mithen 2000). Low volatility of sinalbin hydrolysis products may be responsible

for the absence of these compounds in the headspace of *S. arvensis* and explain why this plant species was less attractive to *C. glomerata*. More detailed studies are necessary to elucidate the importance of GS breakdown products as cues for host-plant selection in parasitoid species that parasitise hosts specialised on brassicaceous plant species.

In summary, we have demonstrated that differences in food plant quality for the host, *P. brassicae*, did not have a negative impact on the development of its larval endoparasitoid, *C. glomerata*. The feeding strategy of the parasitoid larvae, i.e., selectively feeding on haemolymph and fat body, probably enabled the parasitoid to adjust its feeding behaviour without affecting the size or development time of its progeny. In the flight-chamber bioassay, female *C. glomerata* preferred damaged over undamaged conspecific plants and also exhibited a preference for herbivore-damaged *B. nigra* over herbivore-damaged *S. arvensis* plants. Although both plant species are annuals, the phenology of the two plant species differs. *Brassica nigra* usually flowers between June and August whereas *S. arvensis* exhibits two flowering peaks, the first in May-June and the second between September and November. In The Netherlands, *P. brassicae* and *C. glomerata* are primarily bivoltine. The first generation of both the host and the parasitoid coincide with the phenology of *S. arvensis*, whereas the second generation coincides with the phenology of *B. nigra*. It is likely that different generations of *P. brassicae* and *C. glomerata* develop on different brassicaceous plant species. Therefore, it is critically important for *C. glomerata* females to be able to recognise a range of host food plants that may differ considerably in their volatile emission when damaged by hosts. High concentrations of GS breakdown products in the headspace of herbivore-damaged plants may play a role in attracting this parasitoid species. In cultivars of *B. oleracea*, levels of GS are often reduced through the process of artificial selection (Benrey et al. 1998; Harvey et al. 2007a; Gols et al. 2008c). Because of this, the role of GS in indirect defence may be less clear than would be the case with wild populations where levels of allelochemicals are often much higher. We argue that it is important to use wild brassicaceous species to determine the relative role of GS in plant defence against insect herbivores.

Acknowledgments

We would like to thank André Gidding and Leo Koopman for supplying insects, Suzanne Blatt for assisting with the flight chamber bioassays and Tibor Bukovinszky for statistical advice. Roel Wagenaar and Nicole van Dam are acknowledged for conducting and interpreting the GS analyses. This project was supported by the Uyttenboogaart-Eliassen foundation.



7

Comparing direct and indirect plant defences in *Brassica nigra* populations that have been exposed to different selection pressures

by R. Gols, N.M. van Dam, C.E. Raaijmakers,
M. Dicke & J.A. Harvey

Direct and indirect plant defences are usually studied independently. However, it is possible that the same sets of genes regulate both defence strategies in plants, suggesting the possibility of conflicts between them. In this study we examined direct and indirect defences in populations of brown and black mustard (*Brassica juncea* and *B. nigra*). The growth and development of two lepidopteran herbivores, *Pieris brassicae* and *Plutella xylostella*, and their respective hymenopteran endoparasitoids, *Cotesia glomerata* and *Diadegma semiclausum* was monitored on two wild *B. nigra* populations originating in The Netherlands and Sicily (Italy), as well as single cultivated strains of *B. nigra* and *B. juncea*. In addition, attraction of the two parasitoid species to volatiles from plants of the different populations when infested by their respective hosts was compared. The results of this study show that the four mustard populations differentially affected performance (development time and body mass) of the two herbivore species. However, contrasts among the means revealed significant differences in only a few comparisons suggesting that plant quality had only a marginal effect on herbivore and parasitoid performance. In behavioural bioassays, the two parasitoid species were differentially attracted to the four plant populations. Female *D. semiclausum* only discriminated between *B. juncea* and *B. nigra* plants, whereas *C. glomerata* was less attracted to plants of the Sicilian *B. nigra* population. The results of this study suggest that there is no apparent conflict between direct and indirect plant defences. Chemical analysis of foliar glucosinolates and volatile emissions by *P. xylostella*-damaged plants revealed major differences between *B. nigra* and *B. juncea* and only minor differences among the *B. nigra* populations.

Key-words: crucifers, glucosinolates, herbivore-induced plant volatiles, parasitoid

Introduction

Natural plant populations have been demonstrated to differ genetically in important traits, such as defence, over parts of their geographical ranges (Moyes et al. 2000; Brenes-Arguedas and Coley 2005; Zangerl and Berenbaum 2005; Lankau and Strauss 2007). Furthermore, the geographic scale over which detectable differences in these traits can be observed may vary from a few kilometers (Mithen et al. 1995; Moyes et al. 2000) to thousands of kilometers (Zangerl and Berenbaum 2005; Lewis et al. 2006). The evolution of defence and other phenotypic traits in plants is clearly influenced by both top-down (natural enemy) and bottom-up (local environment) selective forces, which in many instances are not mutually exclusive (Hunter and Price 1992). For example, local abiotic conditions, such as wind, ambient air and soil temperature regimes, and rainfall will influence the community of herbivores associated with the plant. Moreover, soil nutrients and other biotic factors including competition with neighboring plants will also strongly affect plant metabolism and result in trade-offs between growth (reproduction) and defence (van Dam et al. 2000; Siemsen et al. 2002; Prudic et al. 2005). Divergent selection pressures are likely to occur in populations that grow at opposite ends of large geographical ranges where environmental conditions may be very different.

Plant defence traits that negatively affect herbivore performance include morphological and chemical adaptations and are collectively known as 'direct defences'. Morphological plant characteristics such as hairs, spines and glossy leaf surfaces hamper herbivore movement on the plant, whereas plant chemicals such as toxins, repellents and digestibility reducers may compromise growth and development of the herbivore. Among the different plant defences, the effects of plant secondary compounds on the development and survival of insect herbivores are well studied (Rosenthal and Berenbaum 1992; Schoonhoven et al. 2005). However, plant-derived compounds have been demonstrated to also affect the performance of natural enemies of insect herbivores such as parasitoids and predators (reviewed by Harvey 2005; Ode 2006).

Many studies have reported that plants also possess chemical and/or structural traits that attract or harbour natural enemies of their herbivores, and which are collectively known as 'indirect defences' (Dicke 1999c). For instance, some plants produce specialised structures, such as domatia, that encourage natural enemies to live on the plants where they function as 'bodyguards' (Grostal and Odowd 1994; Brouat and McKey 2001). Other plants produce sugar-rich substances, such as extra-floral nectar that nourish the herbivore's natural enemies (Wäckers and Bonifay 2004). Lastly, most plants release volatile chemicals in response to herbivore damage, which are attractive

to parasitoids and predators of herbivores (reviewed by Turlings and Benrey 1998; Dicke 1999b; Takabayashi et al. 2006; Heil 2008). However, whether the production of herbivore-induced plant volatiles (HIPV) is an evolutionarily reinforced trait remains the subject of debate (van der Meijden and Klinkhamer 2000; Hoballah and Turlings 2001; Janssen et al. 2002; Heil 2008). If it is, we would expect genetic variation in the production of volatile blends among individual plants and between different plant populations, as has been demonstrated with direct defence. This is because of habitat-related differences in the intensity of herbivore attack or in which the reliability of 'alarm signals' to natural enemies varies (van der Meijden and Klinkhamer, 2000).

Importantly, direct and indirect defences may not act independently but instead genes regulating the expression of different defence processes may interact. For instance, the plant hormone jasmonic acid plays an important role in the induction of both direct and indirect plant defences (Dicke and van Poecke 2002) and cross talk between signalling pathways may control the opposing effects of different defence traits (De Vos et al. 2005). Consequently, a plant with high levels of direct defences may also have high levels of indirect defences and *vice versa*. On the other hand, plants may also be able to differentially control the expression of their direct and indirect defences (Kahl et al. 2000).

Organisms towards the terminal end of the food chain obtain their nutrition indirectly from the food plants of their hosts or prey. A conflict between direct and indirect defence may occur when natural enemies of the herbivore, such as parasitoids or predators, are attracted by signals emitted by plants that are toxic for their own development (Havill and Raffa 2000). One of the most challenging issues in the study of plant-insect interactions is therefore to determine how this potential conflict is resolved. However, it has been a longstanding practice in ecology to study direct and indirect plant defences independently (Hunter 2003). When conflicts are discussed, this is generally done within a narrow framework and often studiously avoids the perplexing question of opposing selective forces acting on direct and indirect defence strategies in plants. This could lead to misleading conclusions being generated about factors underlying the evolution of defence strategies in plants.

This study examines direct and indirect defences in populations of black and brown mustard (*Brassica nigra* L. and *B. juncea* L.). The growth and development of two specialist lepidopteran herbivores, *Pieris brassicae* L. and *Plutella xylostella* L., and their respective endoparasitoids, *Cotesia glomerata* L. and *Diadegma semiclausum* Hellén was monitored on two wild *B. nigra* populations originating in The Netherlands and Sicily (Italy), as well as single cultivated strains of *B. nigra* and *B. juncea*. *Brassica nigra* is a widespread weedy annual over much

of Eurasia and is also cultivated as a condiment and oilseed crop. Plant defences in the *B. nigra* cultivar might differ from those in the two wild populations due to the process of artificial selection. *B. juncea* is a hybrid of *B. nigra* and oilseed rape, *B. rapa*. This species was included because it was expected to express some defence traits of *B. nigra* whereas other traits were expected to differ. The following hypotheses were tested: 1) direct and indirect plant defences vary among populations of *B. nigra* that have been exposed to different selection pressures, either naturally or artificially. 2) Differences in herbivore and parasitoid performance and parasitoid behaviour reflect concomitant differences in plant chemistry. 3) Direct and indirect defences are compatible. All plant species belonging to the family of Brassicaceae are able to biosynthesise glucosinolates (β -thioglucoside-N-hydroxysulfates) (Fahey et al. 2001; Mithen 2001b). Glucosinolates (hereafter GS) and their breakdown products play an important role in defence against pathogens and insect herbivores (Chew 1988; Rask et al. 2000). We measured levels of GS as an indicator of direct plant defence and volatile emissions as an indicator of indirect defence.

Materials and methods

Plants

The natural range of *B. nigra* or black mustard extends from the Mediterranean region to the Middle East and Central Asia (Gómez-Campo and Prakash 1999). Seeds were collected by early civilisations as a condiment but also for medicinal purposes (Gómez-Campo and Prakash 1999). *Brassica nigra* seeds were collected in The Netherlands (hereafter NL-wild population), 4 km west of Wageningen. A second wild population was sampled on western Sicily, Italy, near the village of Scopello (hereafter IT-wild). Seeds of a German cultivar of *B. nigra* ('Junius', hereafter CV) were obtained from a seed bank (Center for Genetic Resources, Wageningen, The Netherlands). The production of *B. nigra* has diminished in favour of brown or Indian mustard, *B. juncea*, a hybrid of *B. nigra* and *B. rapae*. *Brassica juncea* is believed to originate from the Middle East and Central Asia (Gómez-Campo and Prakash 1999) where both wild forms of its parent species occur. Seeds of a cultivar of *B. juncea* were bought at a local market on Zanzibar, Africa. We also grew Brussels sprouts plants (*B. oleracea* L. gemmifera cv. Cyrus), which were used for insect rearing.

Plants were grown in a greenhouse (25 ± 5 °C, 40–80% relative humidity and a photoperiod of at least 16 h). If the light dropped below 500 $\mu\text{mol photons/m}^2/\text{sec}$ during the 16-hr photoperiod, supplementary illumination was applied by high-

pressure mercury lamps. *Brassica nigra* seeds were grown individually in 1.5-l pots filled with potting soil ("Lentse potgrond" no. 4, Lent, The Netherlands). When the plants were 4 weeks old, they were used for oviposition by *P. xylostella* and *P. brassicae* or in flight-chamber bioassays.

Insects

Plutella xylostella is a major pest species causing worldwide damage to cabbage crops (Talekar and Shelton 1993). It is a solitary herbivore, i.e. eggs are laid singly. One of its solitary larval endoparasitoids is *D. semiclausum*. This species can successfully develop in all larval stages of its host. Larvae of *D. semiclausum* consume virtually all tissues of *P. xylostella* larvae before spinning a cocoon on the food plant.

Pieris brassicae is also a specialist herbivore on brassicaceous plant species and like *P. xylostella* is considered to be a pest of cabbage crops over much of its range. The female butterfly deposits eggs in clutches and the larvae feed gregariously. *Cotesia glomerata* is a gregarious larval endoparasitoid of larvae belonging to a few genera of the Pieridae (Brodeur and Vet 1995). Adult female wasps attack first to third instar larvae of *P. brassicae* and their larvae feed primarily on host haemolymph and fat body. Fully grown parasitoid larvae egress from the host caterpillar when it has reached the final (fifth) instar (hereafter L5) and they spin cocoons on the underside of a leaf on the host food plant.

Both herbivore species were originally collected from Brussels sprout fields in the vicinity of Wageningen (The Netherlands) and have been reared in the laboratory for many years. Fresh cultures of the parasitoids are started once every year with field collected individuals. Cultures of the two herbivores were maintained in climate rooms at $22 \pm 2^\circ\text{C}$, 50-70% r.h. and a photophase of 16L:8D. Parasitoids were reared on their respective hosts and kept in a greenhouse or climate rooms under the same conditions as the host (for details on parasitoid rearing see Harvey 2000; Gols et al. 2007)

Development of the herbivores and parasitoids on different plant populations

To obtain larvae of *P. xylostella* for the developmental study with the four different plant populations, one plant of each population was placed in a cage (40 x 40 x 65 cm) with c. 100 *P. xylostella* moths in a 50:50 female to male ratio. After an overnight oviposition period, the moths were removed and the plants were left in the rearing cages for development of the eggs into larvae. When the larvae reached L2, a cohort of between 70 and 90 of them were placed in even distributions over 5 fresh plants of their respective population. This process was repeated for all 4 mustard populations.

A second cohort of 100-110 larvae per plant population was parasitised by *D. semiclausum*. Larvae were parasitised by presenting them individually to mated female wasps in plastic vials on the end of a small brush. Larvae were considered to be parasitised when female wasps were visually observed to have inserted and removed their ovipositor from a host. The parasitised larvae were then transferred to 9 new plants from their respective experimental host-plant population. Each female wasp was allowed to parasitise up to 10 individual hosts. When both healthy and parasitised larvae reached L4, pieces of corrugated cardboard (5 x 20 cm) were placed on top of the plants, as the larvae exhibit a tendency to seek sheltered places in which to construct cocoons. Herbivore and parasitoid pupae were placed individually into labelled glass vials until adult eclosion.

The development of *P. brassicae* and *C. glomerata* were also monitored on the four different plant populations. Individual plants from each of the populations were placed in the rearing cage with adult butterflies for approximately 3 h. After this time, plants generally contained 4-5 egg clutches per plant. Plants were then removed from the cages and left in the rearing room for the eggs to hatch and the larvae to develop. When the larvae reached L2, one cohort (unparasitised) of 40-45 randomly selected larvae was transferred to two new plants of the same plant population that they had been feeding on previously. Larvae were equally divided over the two plants and placed together on one leaf, because young *P. brassicae* larvae feed gregariously. A second cohort of 40-45 larvae were randomly selected from the rearing cages for parasitism by *C. glomerata* females. The procedure for parasitism was the same as that described for *P. xylostella* and *D. semiclausum*. Larvae were presented individually to mated female *C. glomerata* in plastic vials on the end of a small brush. Larvae were considered to be parasitised when the wasps had inserted their ovipositor for at least 10 s, during which time a full brood is deposited into the host (Harvey 2000). Each female wasp was allowed to parasitise up to 10 individual hosts. Parasitised larvae were then placed onto the leaves of two new plants from the same population that they had been feeding on previously. To ensure that the larvae received sufficient food, extra plants were added as required. In the end, this meant that up to 12 plants were used per population. After pupation, *P. brassicae* pupae (from the unparasitised cohort) were transferred to small plastic containers (17 x 12 x 6 cm, 10 pupae per container) lined with filter paper. *Cotesia glomerata* cocoons were transferred to labelled Petri dishes (9.5 cm in diameter) containing one brood per dish.

Vials and dishes were checked every 2 hours and the hour and date of emergence were recorded. The egg-to-adult development time of all herbivores and parasitoids that emerged was recorded. Development time was determined in hours (for parasitoids) and in days (for herbivores). Newly emerged adults

were frozen and dried for 3 days in an oven at 80 °C to obtain dry mass data and were then weighed on a Cahn C-33 microbalance (Cahn Instruments, USA). For *C. glomerata*, we determined the mean dry mass and egg-to-adult development time per brood for males and females separately.

Experiments were conducted in two greenhouses, one for each herbivore-parasitoid combination, under the same conditions as the plants. Plants from the same population with the same insect treatment were placed together. The different treatments were spatially separated and randomly positioned in a greenhouse compartment, but were all placed in a similar position relative to the light sources to minimise the effect of any microclimatic differences between the plant populations and treatments.

Glucosinolate analyses of leaf tissues damaged by herbivores

From each plant population, leaf samples for GS analysis were taken from plants damaged by both parasitised and unparasitised *P. xylostella* larvae. Leaf samples were also taken from plants from the NL-wild population that had been damaged by *P. brassicae* larvae. The herbivore larvae had been feeding on the plants for 7 days when the samples were collected. To standardise the samples, the 6th and 7th leaves moving down from the top of the plant were collected. One leaf was sampled per plant (12-14 leaves per population) and all sampled leaves were checked to ensure that they had moderate traces of feeding damage. During the developmental studies, plants from the *B. juncea* population were flowering. Therefore, we also took one sample of pooled flower petals from *B. juncea* plants damaged by *P. xylostella*. Leaves were cut off using a razor blade and immediately stored at -20 °C. Samples were freeze-dried and pulverised using a mortar and pestle. Fifty mg aliquots were weighed in 2 ml centrifuge tubes.

GS were extracted and purified as described in van Dam et al. (2004). GS were separated on a reverse phase C-18 column (Alltima C-18, 3µm, 150 x 4.6 mm, Alltech, Deerfield, IL, USA) on HPLC (DIONEX, Sunnyvale, CA, USA) with an acetonitrile water gradient. Detection was performed with a DIONEX PDA-100 Photodiode array detector set to scan from 200 to 350 nm. For quantification, sinigrin (Sigma, St. Louis, IL, USA) was used as an external standard. Peaks were integrated at 229 nm for which standard response factors have been defined (EC 1990). The different GS were identified based on their retention times and UV spectra compared to those of pure compounds, sinigrin (Sigma, St. Louis, IL, USA), glucotropaeolin, sinalbin, and glucobrassicin (kindly provided by M. Reichelt, MPI Chemical Ecology, Jena, Germany), or compared with a certified oil seed reference (EC

Community Bureau of Reference BCR-367R, Fluka, Buchs, Switzerland)

Parasitoid flight responses to four plant populations infested with their respective hosts

The flight response of female *C. glomerata* and *D. semiclausum* wasps to plants infested with their respective hosts was tested in a greenhouse (22-25 °C, 70% r.h.) using a tent-like structure (330 x 175 x 280 cm), which was made out of white nylon sheets (for details see Wiskerke and Vet 1994). The greenhouse was continuously ventilated which created a turbulent air-current inside the tent. Pairwise comparisons were made for all 4 different plant populations i.e. in total six pairwise comparisons were made for each of the two host-parasitoid combinations. *Cotesia glomerata* females were collected from the main culture by placing a leaf with L1 and L2 *P. brassicae* larvae in a rearing cage for less than a minute. Females were collected from this leaf with a fine paintbrush and transferred to 5 ml-vials, which were covered with cotton wool. Wasps were used in the bioassay within 10 min after collection. Oviposition experience enhances the motivation of the wasps to forage for hosts. With *D. semiclausum* we used mated females that were between 4 and 7 days old and had not been previously exposed to hosts. Prior to release in the bioassays, the wasps were collected from the cage in 5-ml vials and presented with a small piece of cabbage leaf with host feeding damage but without the host itself or any of its products. Both wasp species had never experienced *B. nigra* or *B. juncea* volatiles before.

In the flight chamber, two host-infested plants, each from a different population, were placed on a table 60 cm apart. Plants were infested with fifteen L1 *P. brassicae* or ten L3 *P. xylostella* larvae on the day prior to the bioassay. Each set of plants had received a similar amount of herbivore damage based on visual inspection. Females were released in vials approximately 100 cm away from the two host-infested plants. Since wasps did not leave the vial instantaneously after removal of the cotton wool, about 10 vials were put on the table simultaneously. Wasps were allowed to fly freely within the tent. As soon as a wasp landed on one of the two plants, it was collected and the population on which it had landed was recorded. If some of the wasps that were initially released had not alighted on one of the plants within 30 minutes, they were excluded from the analyses and additional wasps were released. A total of 10-14 wasps were recorded per combination of two plants. Each wasp was only used once. To control for any bias in the position of the plant on the table, the position of the plants was changed after 2-3 wasps had landed on the plants. Each plant combination was tested 13 times with newly prepared plants. In total, 826 *D. semiclausum* and 841 *C. glomerata* females were tested with 2 x 156 plants

over a 5-month period. To determine whether the amount of leaf damage differed among the populations, we estimated the area of removed leaf tissue after 24 h of herbivore feeding using mm-paper for 10-12 plants of each plant population and herbivore species combination.

Headspace analysis of herbivore infested plants: collection of volatiles

As an indicator of indirect defence, the headspace of herbivore-damaged plants was collected and analysed by means of GS-MS. A number of *P. xylostella*-infested plants ($n = 5$ per plant population) were used for headspace collection after the plants had been tested in the flight response bioassay. An entire host-infested plant, including soil, which was contained in aluminum foil, was transferred to a clean 35-litre glass collection container. Pressurised air was filtered over silica gel, molecular sieves (4Å, Sigma, St Louis MO, USA) and activated charcoal before entering the sampling container. Prior to sampling, the container was purged for 1 h at 10 l/h. Subsequently, the headspace volatiles were trapped on 90 mg Tenax TA for 4 hrs at a flow of 70 ml/min. Volatiles were also collected ($n = 4$) from a collection container containing a piece of aluminum foil with water. Volatile compounds detected in these control samples were excluded from the data obtained for the plant samples.

The collected volatiles were released from the Tenax traps by heating in a thermodesorption cold trap unit (Markes, UK) at 200 °C for 10 min and flushing with a He flow of 30 ml/min. The released compounds were cryofocused in a coldtrap at a temperature of -10 °C. Volatiles were injected in splitless mode into the RTX-5 Silms GC column (Restec, 30 m x 0.32 mm ID, 0.33 µm film thickness) by heating of the cold trap to 270 °C. After an initial column temperature of 40 °C for 2 min, the temperature was raised to 90 °C at 3 °C/min, then to 165 °C at 2 °C/min, and subsequently to 250 °C at 15 °C/min. The column was directly coupled to the ion source of a Finnigan quadrupole mass spectrometer, which was operating in the 70 eV EI ionisation mode and scanning from mass 33 to 300 at 3 scans/sec. Compounds were identified by comparison of the mass spectra with those in the NIST 2002 (version 2a) and Wiley 7 edition spectral libraries and by checking the retention indices.

Statistical analysis

Data on adult dry mass and development time of parasitised and unparasitised hosts respectively were analysed using 2-way ANOVA with plant population, sex and their interactions as factors. The Tukey-Kramer method was used for multiple comparisons of means. Differences in GS concentrations among the populations were also analysed by means of ANOVA (one-way) followed by

Tukey-Kramer multiple comparisons of the means. A previous study revealed that GS levels in leaf tissues did not differ between plants damaged by parasitised and unparasitised *P. xylostella* larvae (Gols et al. 2008b). Therefore, data from plants with either parasitised or unparasitised larvae were pooled per plant population.

To statistically analyze the parasitoid landing preferences, we calculated the proportion of wasps that landed on either plant population in each bioassay. We used two-tailed *Z*-tests to determine whether the mean proportion differed from $\mu=0.5$ (H_0 =both plant population are equally attractive). To see whether population A differed in attractiveness irrespective of results with the other populations (B, C and D), bioassays with population A were pooled and a *Z*-test was used to determine whether the mean proportion ($n = 39$) differed from $\mu = 0.5$.

Kruskal-Wallis tests were used for each of the volatile chemicals, measured as peak area / g fresh weight, to reveal significant differences in volatiles emission among the four plant populations.

Results

Development of *P. xylostella* and *D. semiclausum* on four different plant populations

Females of *P. xylostella* readily accepted the four different plant populations as suitable plants for oviposition. There was a significant effect of plant population on egg-to-adult development time of *P. xylostella* ($F_{3,262} = 10$, $P<0.001$; Fig. 1A). Adult *P. xylostella* attained similar body masses on all four populations ($F_{3,260} = 1.2$, $P = 0.31$; Fig. 1B), with the females being more than twice as heavy as the males ($F_{1,260} = 1896$, $P<0.001$). For the parasitoid *D. semiclausum* reared from *P. xylostella*, plant population had a significant effect on egg-to-adult development time ($F_{3,284} = 8.9$, $P<0.001$; Fig. 2A) and adult dry mass ($F_{3,282} = 2.7$, $P = 0.04$; Fig. 2B). The male wasps emerging from hosts reared on *B. juncea* developed faster than those emerging on the three *B. nigra* populations on which development time was similar (Fig. 2A). The largest male wasps developed on *B. juncea* and were about 10% heavier than male wasps from the *B. nigra* cultivar which produced the wasps with the lowest biomass. Females obtained significantly larger dry masses than males ($F_{1,282} = 125$, $P<0.001$; Fig. 2B).

Development of *P. brassicae* and *C. glomerata* on four different plant populations

Females of *P. brassicae* also readily accepted the four different plant populations as host plants for oviposition. Plant population

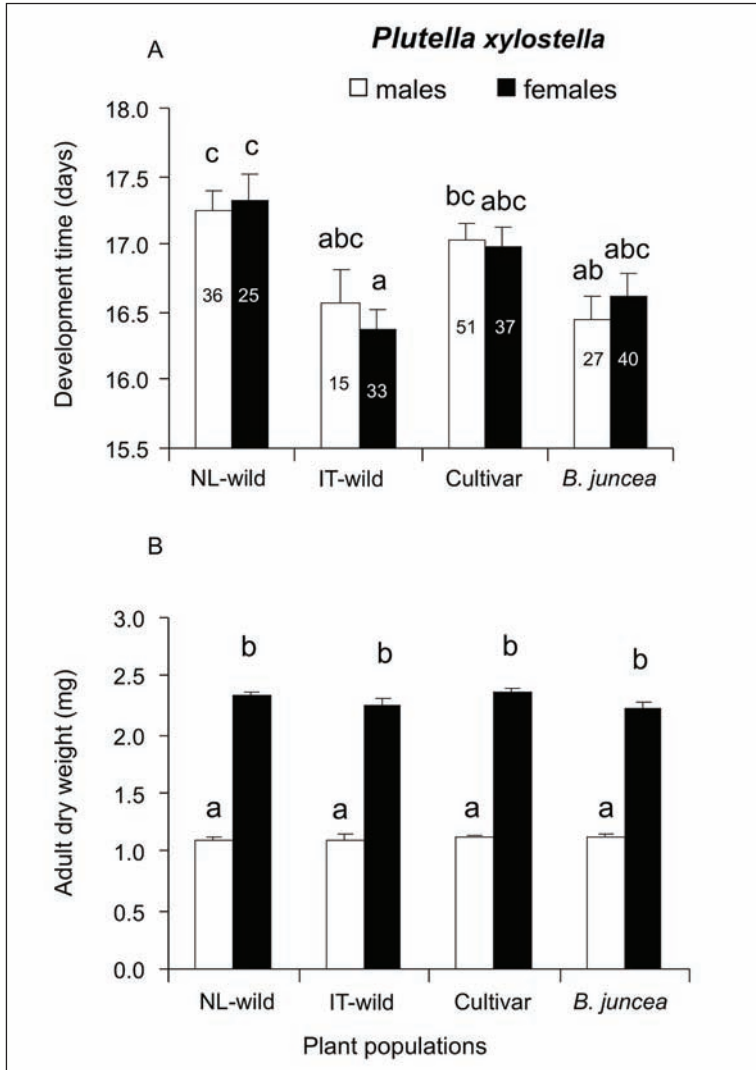
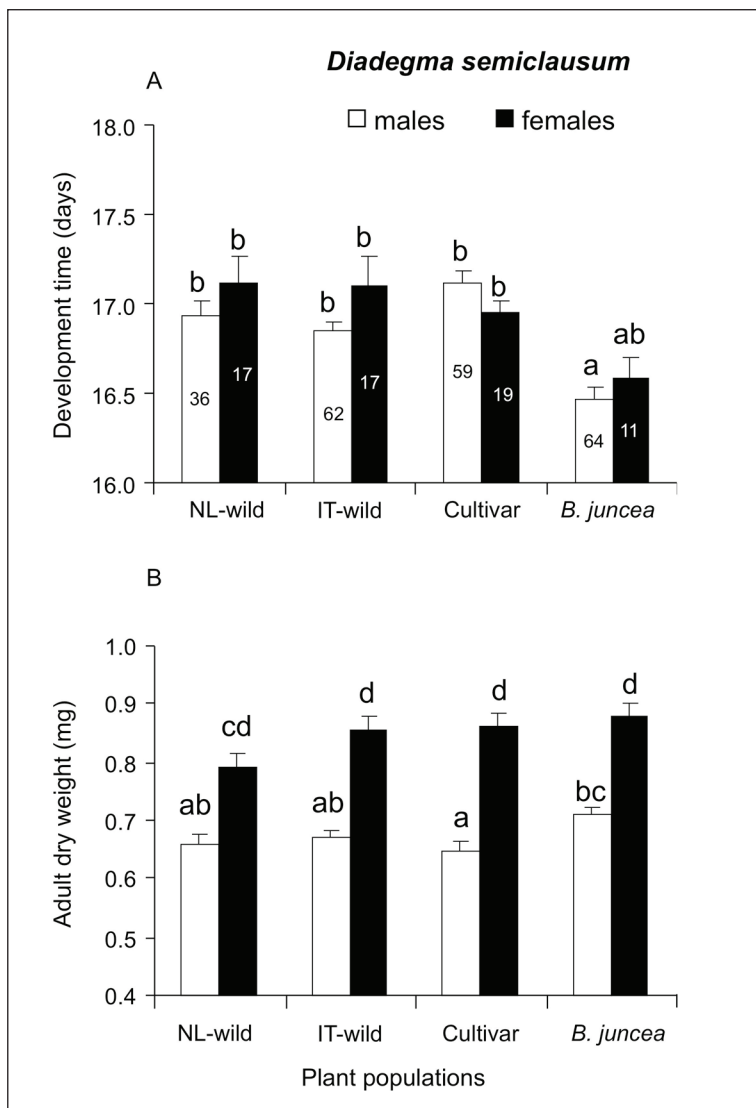


Figure 1: Egg-to-adult development time in days (A) and dry mass in mg (B) of male (white bars) and female (black bars) *P. xylostella* moths that had been reared on three different *B. nigra* and one *B. juncea* population. Bars (mean + SE) with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means). Numbers in bars denote number individuals (*n*). Note that axes do not start at zero.

affected both egg-to-adult development time ($F_{3,161} = 15.6$, $P < 0.001$) and adult dry mass ($F_{3,161} = 13.1$, $P < 0.001$) of *P. brassicae* (Fig. 3). Butterflies that had developed on *B. juncea* were the heaviest but also tended to have longer development times, although this was only significant for males. *Pieris brassicae* had similar egg-to-adult development times and adults attained similar body masses on the three *B. nigra* populations (Fig. 3). Female butterflies generally developed faster ($F_{1,261} = 14.6$, $P < 0.001$) and were heavier ($F_{1,161} = 51$, $P < 0.001$) than male butterflies.

As with *P. brassicae*, plant population significantly affected egg-to-adult development time ($F_{3,334} = 18.7$, $P < 0.001$) and adult dry mass ($F_{3,333} = 24.4$, $P < 0.001$) of the parasitoid, *C. glomerata* (Fig. 4). However, performance of *C. glomerata* on the four plant populations did not reflect performance of

Figure 2: Egg-to-adult development time in days (A) and dry mass in mg (B) of male (white bars) and female (black bars) *D. semiclausum* wasps that had been reared on three different *B. nigra* and one *B. juncea* population. Bars (mean + SE) with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means). Numbers in bars denote number of individuals (*n*). Note that axes do not start at zero.



unparasitised *P. brassicae*. Parasitoids developed fastest on *B. juncea*, which was the population on which development time of unparasitised hosts was the longest. Furthermore, adult wasps were the smallest when reared from hosts on IT-wild plants and obtained similar body masses on the other three populations. This contrasts with *P. brassicae*, where adult mass did not differ on the three *B. nigra* populations, but was highest on *B. juncea*. The parasitoid exhibited protandry, whereby female wasps took longer to complete their development than males ($F_{1,334} = 57.3$, $P < 0.001$), but females were also larger in terms of adult body mass ($F_{1,333} = 186$, $P < 0.001$).

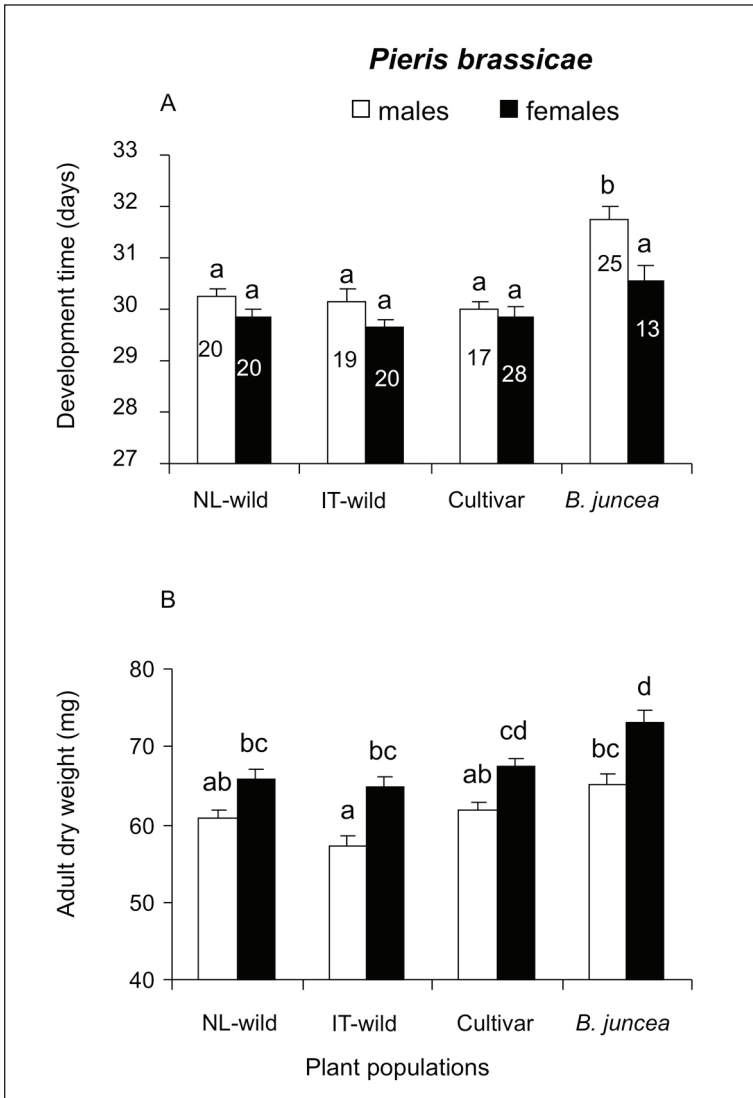


Figure 3: Egg-to-adult development time in days (A) and dry mass in mg (B) of male (white bars) and female (black bars) *P. rapae* butterflies that had been reared on three different *B. nigra* and one *B. juncea* population. Bars (mean + SE) with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means). Numbers in bars denote number of individuals (*n*). Note that axes do not start at zero.

Glucosinolate analyses of leaf tissues damaged by herbivores

GS analyses revealed mainly quantitative and some qualitative differences among the *Brassica* populations (Table 1). Sinigrin was the dominant GS in the *B. nigra* populations and contributed on average more than 98% of the GS content, whereas in *B. juncea* gluconapin was the dominant GS (73% of the total GS content) followed by sinigrin (23%). In *B. juncea*, levels of sinigrin and gluconapin were higher in flower petals than in leaves: 3.4 and 2.4 times, respectively. Although levels of the other GS compounds differed significantly among some of the plant populations, none of the measured GS exceeded concentrations of 0.5 $\mu\text{mol/g DW}$ in any of the samples. Levels of the different GS were similar in plants infested with *P. xylostella* and *P. brassicae* (Table 1). Total

amounts of GS were significantly higher in IT-wild plants than in the two cultivars.

Tabel 1: GS concentrations (mean $\pm$ SE $\mu\text{mol/g}$ DW) in leaf tissues collected from three *B. nigra* and one *B. juncea* population damaged by *P. xylostella* larvae. Additionally, GS concentrations were determined in leaves from the NL-wild population when damaged by *P. brassicae* larvae and in one pooled sample with flower petals of *B. juncea* plants. The ANOVA results on GS concentrations are given as the *F*-value followed by the *P*-value between brackets. Different letters within a GS column denote populations that have different mean concentrations (Tukey-Kramer multiple comparisons among means). SIN=sinigrin, GNA=gluconapin, GBC=glucobrassicin, NAS, gluconasturtiin, TOT=total GS content

Pop	<i>n</i>	SIN	GNA	GBC	NAS	TOT
NL-wild(<i>Plutella</i>)	13	32.9 $\pm$ 3.1bc	0.06 $\pm$ 0.02a	0.030 $\pm$ 0.004a	0.26 $\pm$ 0.04b	33.2 $\pm$ 3.1ab
NL-wild(<i>Pieris</i>)	13	35.4 $\pm$ 4.0bc	0.05 $\pm$ 0.01a	0.024 $\pm$ 0.003a	0.25 $\pm$ 0.04ab	35.4 $\pm$ 4.0ab
IT-wild(<i>Plutella</i>)	12	44.3 $\pm$ 3.7c	0.07 $\pm$ 0.02a	0.074 $\pm$ 0.011b	0.17 $\pm$ 0.03ab	44.3 $\pm$ 3.7b ¹²
CV(<i>Plutella</i>)	14	30.0 $\pm$ 3.6b	0.02 $\pm$ 0.02a	0.039 $\pm$ 0.006a	0.15 $\pm$ 0.02a	30.2 $\pm$ 3.6a ¹²
<i>B. juncea</i> (<i>Plutella</i>)	14	7.3 $\pm$ 1.3a	21.7 $\pm$ 1.6b	0.029 $\pm$ 0.003a	0.16 $\pm$ 0.02a	29.6 $\pm$ 2.6a ²³
ANOVA d.f.=4,61		3.17(0.02)	1713(<0.001)	10.5(<0.001)	2.82(0.03)	3.17(0.02)
<i>B. juncea</i> -flowers	1	25.2	53.0	0.08	0.50	79.4 ¹²⁴

¹ A few samples contained low levels (<0.06 $\mu\text{mol/g}$ DW per samples) of 4-hydroxyglucobrassicin, which is included in the total.

² A few samples contained low levels (<0.06 $\mu\text{mol/g}$ DW per samples) of 4-methoxyglucobrassicin, which is included in the total.

³ Total GS included glucobrassicinapin 0.44 $\pm$ 0.11 $\mu\text{mol/g}$ DW and neoglucobrassicin 0.012 $\pm$ 0.002 $\mu\text{mol/g}$ DW

⁴ Total GS included glucobrassicinapin 0.62 $\mu\text{mol/g}$ DW.

Parasitoid flight responses to four plant populations infested with their respective hosts

Plant population did not affect the amount of leaf area removed by the host larvae (*P. xylostella*: $F_{3,42} = 2.14$, $P = 0.11$; *P. brassicae*: $F_{3,44} = 1.83$, $P = 0.16$). Both species of parasitoids discriminated between some of the different plant populations (Fig. 5). Significantly more *D. semiclausum* females landed on *B. juncea* plants when offered with a NL-wild or a cultivated *B. nigra* plant (Fig. 5A). The overall preference for *B. juncea* was therefore found to be highly significant ($t_{38} = 3.95$, $P < 0.001$). No significant differences were found in landing preference of *D. semiclausum* in any of the other population comparisons (Fig. 5A). Preference for a specific plant population by female *C. glomerata* was more idiosyncratic (Fig. 5B). Female wasps of this species preferred NL-wild over IT-wild and the *B. nigra* cultivar over NL-wild. However, when all the data with IT-wild plants were pooled, *C. glomerata* females were significantly less attracted to this plant population than to the others ($t_{38} = 3.31$, $P < 0.002$).

Headspace analysis of herbivore infested plants

In total, 17 different compounds were detected in the headspace of the four different plant populations (Fig. 6). Fourteen compounds were found in all for populations. Ketones were quantitatively the most abundant group (49% of total emission in *B. juncea*, 65% in NL-wild, 66% in IT-wild, and 59% in the *B. nigra* cultivar). Allyl isothiocyanate, a hydrolysis product of sinigrin, was detected in

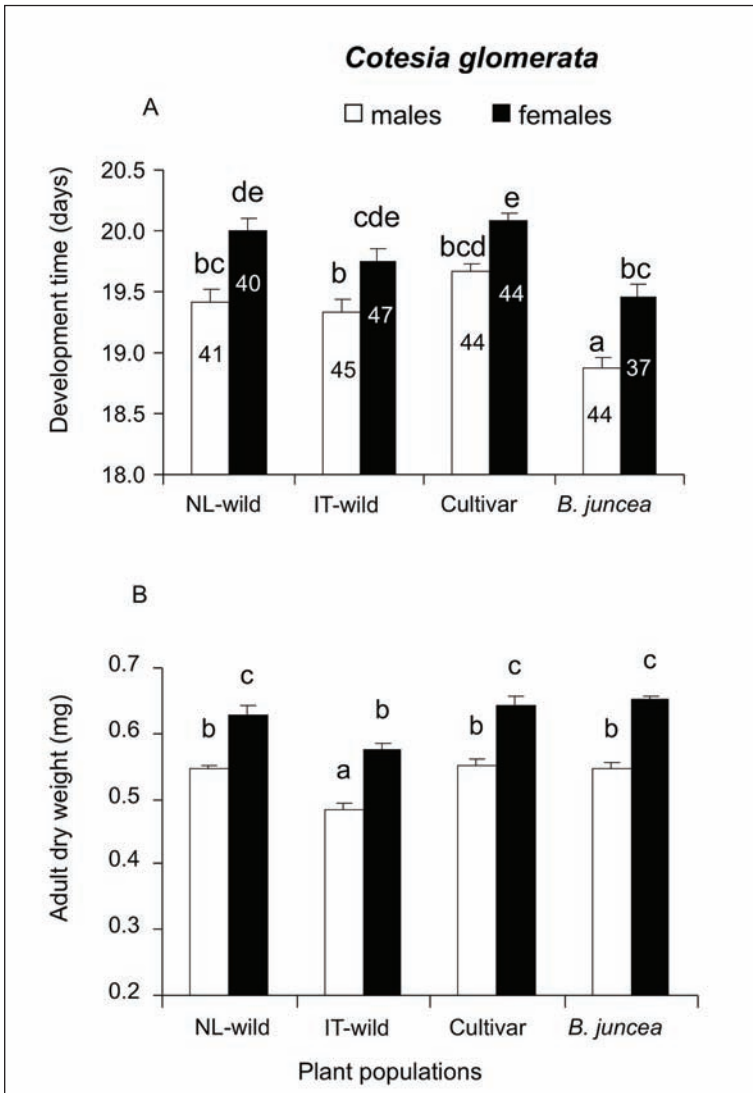


Figure 4: Egg-to-adult development time in days (A) and dry mass in mg (B) of male (white bars) and female (black bars) *C. glomerata* wasps that had been reared on three different *B. nigra* and one *B. juncea* population. Wasps were pooled per brood for each sex. Bars (mean + SE) with the same letter are not significantly different (Tukey-Kramer test for multiple comparisons among means). For each brood (= offspring per host), the mean development time and body mass was determined for males and females separately. Numbers in bars denote number of broods (*n*). Note that axes do not start at zero.

marginally lower concentrations in *B. juncea* than in the three *B. nigra* populations. In contrast 3-butenyl isothiocyanate as well as 3-methyl-3-butenenitrile, which are both hydrolysis products of gluconapin, were only detected in the headspace of *B. juncea* plants. The nitrile, 2-methyl-butanenitrile, was only emitted NL-wild and IT-wild plants. Plants also produced the following three terpenoids: (*E*)- β -ocimene, (*Z*)- β -ocimene and (3*E*)-4,8-dimethyl-1,3,7- nonatriene. The former two were only detected in *B. juncea* plants.

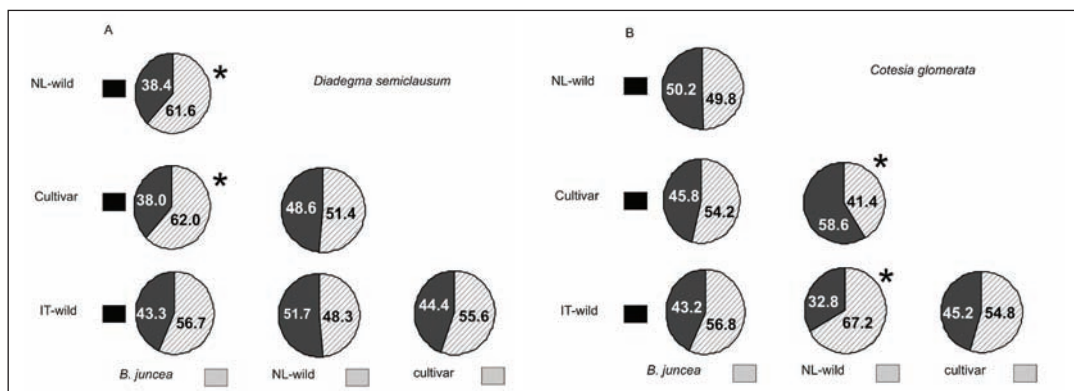


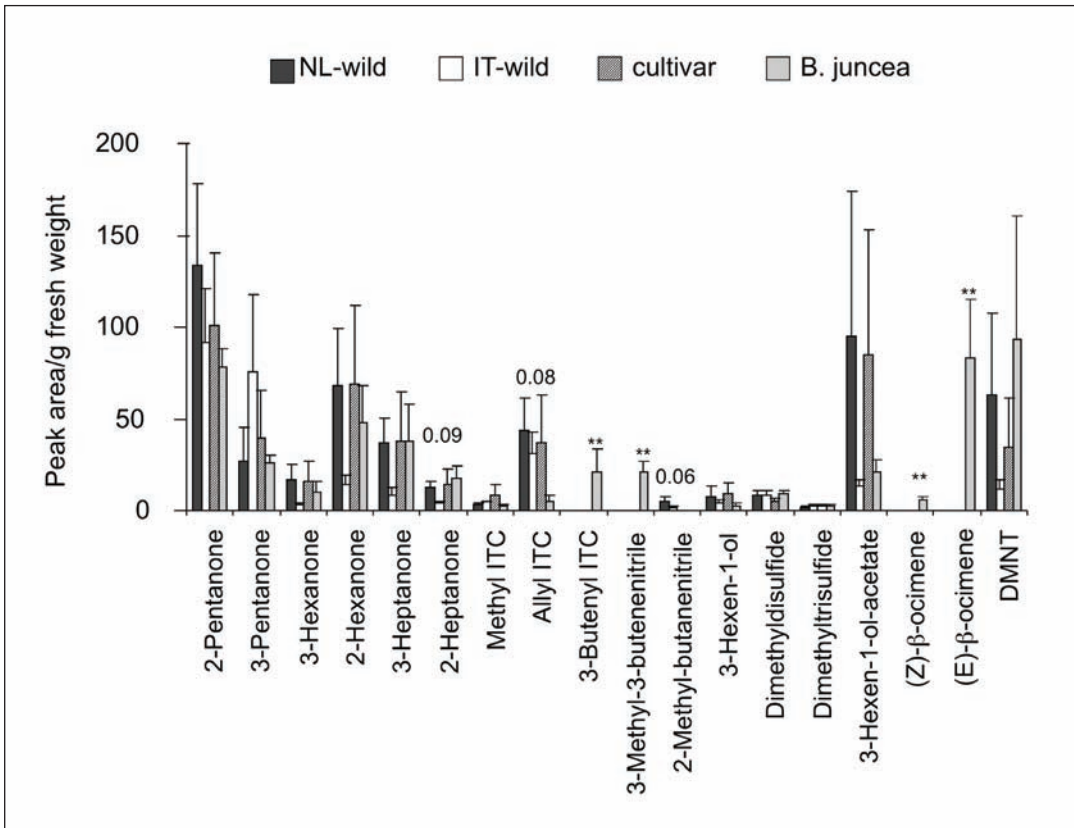
Figure 5: Landing preference of *C. glomerata* (A) and *D. semiclausum* females (B) in a dual choice situation with two host-infested plants each of a different *Brassica* population. Plants were infested with 15 L1 *P. brassicae* (A) or 10 L3 *P. xylostella* larvae (B) on the day prior to the bioassay. Each pie graph represents the mean result of 13 bioassays with different sets of plants, which were each tested with 10-14 wasps. The numbers within the pie give the mean percentage of wasps that landed on each plant. Asterisks indicate bioassays in which wasps preferred one population over the other ($P < 0.05$).

Discussion

The results of this study show that the four mustard populations differentially affected performance (development time and body mass) of the two herbivore species, *P. xylostella* and *P. brassicae* and their larval endoparasitoids, *D. semiclausum* and *C. glomerata*, respectively. However, contrasts among the means revealed significant differences in only a few cases, suggesting that plant quality had only a marginal effect on herbivore and parasitoid performance. GS and their breakdown products have been shown to play an important role in defence against, especially generalist, insect herbivores (Chew 1988; Rask et al. 2000). Here, GS concentrations in leaf tissues were measured as an indicator of direct plant defence. GS analysis revealed major quantitative differences between *B. nigra* and *B. juncea* and only minor quantitative differences among the *B. nigra* populations. *Brassica juncea* contained relatively high levels of gluconapin and relatively low levels of sinigrin, whereas in the *B. nigra* populations, sinigrin formed more than 98 % of the GS content. GS patterns in *B. nigra* and *B. juncea* were similar to those reported in earlier studies (Traw 2002; Bellostas et al. 2007; Smallegange et al. 2007), although the ratio of sinigrin and gluconapin can differ considerably among *B. juncea* cultivars (Li et al. 2000; Müller and Sieling 2006). Clearly artificial selection in *B. nigra* has not resulted in the dramatic changes in GS content as seen in other brassicaceous crop plants such as cabbage (*B. oleracea*) varieties (Rosa 1999; Moyes et al. 2000; Gols et al. 2008c). Furthermore, because it is a hybrid species, the expression of GS in *B. juncea* clearly reflects GS composition of its 'parent' plants, *B. nigra* and *B. rapa*.

Interestingly, the two wild populations, which originated from different geographical locations (The Netherlands and Sicily), did not differ in the composition of GS in their leaves. Several studies have shown that natural plant populations vary considerably in the levels of their secondary plant compounds

(Vrieling et al. 1991; Moyes et al. 2000; Brenes-Arguedas and Coley 2005; Zangerl and Berenbaum 2005). This variation is considered to be the result of frequency-dependent selection by a range of attackers that most commonly occur in their locality. For example, Zangerl and Berenbaum (2005) demonstrated that levels of toxic furanocoumarins increased in the invasive weed, the wild parsnip (*Pastinaca sativa*), after the introduction of its major herbivore, the parsnip webworm (*Depressaria pastinacella*).



Tritrophic interactions involving aphids defended by ants on ragwort (*Senecio jacobaea*) and temporal fluctuations in selection pressure by a specialist herbivore (*Tyria jacobaeae*) could explain the maintenance of genetic variation in levels of allelochemicals in natural plant populations (Vrieling et al. 1991). Aphids and ants only are found on plants with low levels of pyrrolizidine alkaloids. In years with high herbivore pressure only those plants with aphids and ants escape total defoliation (Vrieling et al. 1991). Our results suggest that selection on differentiation in GS content in *B. nigra* populations is not very strong. Alternatively, *B. nigra* tolerates moderate levels of herbivory by specialists (Meyer 2000) and may even compensate for tissue removal (Strauss and Agrawal 1999; Blatt et al. 2008).

Figure 6: Composition of the volatile blend emitted by individual plants ($n = 5$) from three different *B. nigra* and one *B. juncea* population. The day prior to volatile sampling, plants were infested with 10 L3 *P. xylostella* larvae, which remained on the plant during volatile collection. Each bar depicts mean peak area / g fresh weight of above-ground tissue. Compounds of which the emitted concentrations differed significantly among the populations are marked with an asterisks ($P < 0.05$) or the P -value when significance levels are marginal. DMNT: (3E)-4,8-dimethyl-1,3,7-nonatriene.

Specialist insect herbivores that are adapted to feed on GS-containing plants are considered to be less affected by the adverse effects of GS than generalists. Both herbivores used in this study, *P. brassicae* and *P. xylostella*, are specialists that are able to efficiently detoxify and excrete GS (Ratzka et al. 2002; Wittstock et al. 2004; Agerbirk et al. 2006). However, development of even specialised insect herbivores can be compromised by high levels of GS hydrolysis products (Li et al. 2000; Agrawal and Kurashige 2003). Our results suggest that levels of GS in the different mustard populations were below the threshold levels that are toxic to *P. brassicae* and *P. xylostella* (see also (Smallegange et al. 2007)).

Thus far, most studies examining possible conflicts in plant defence traits have concentrated on conflicts occurring within, as opposed to between traits. For instance, it has been shown that volatiles released by plants damaged by herbivore species may be used by other herbivores searching for oviposition sites (Bolter et al. 1997; Renwick et al. 2006; Halitschke et al. 2008). However, potential conflicts between direct and indirect plant defences have received much less attention. A conflict may arise when plant allelochemicals negatively affect the development and realised fitness of natural enemies such as predators or parasitoids as mediated through their host or prey. Many larval endoparasitoids use herbivore-induced plant volatiles to locate host-infested plants (Dicke 1999b; Turlings et al. 2002; Heil 2008). At the same time, several workers have reported negative physiological effects of plant-derived compounds on parasitoid performance (Campbell and Duffey 1979; Barbosa et al. 1986). A conflict may arise when highly toxic plant species or genotypes are attractive to parasitoids.

However, no evidence of a conflict was found in the associations studied here. Since both herbivores used in this study efficiently excrete GS ingested with the food, exposure to GS or their breakdown product is expected to be low for the larval endoparasitoids of these herbivores. This was confirmed in a study by Müller et al. (2003) who demonstrated that the haemolymph of *P. brassicae* larvae that had been feeding on GS-containing plants contained only traces of GS. Furthermore, both parasitoids are specialists and are probably adapted to cope with GS that may be ingested during larval development. Conflicts in plant defence strategies are probably much more apparent in associations involving herbivores and less-well adapted generalist natural enemies (Vrieling et al. 1991; Gols et al. 2008b).

Both parasitoid species used in this study discriminate between volatiles emitted by host-damaged versus undamaged conspecific plants (Steinberg et al. 1993; Ohara et al. 2003b), as well as between volatiles emitted by different brassicaceous plant species (Bukovinszky et al. 2005; Gols et al. 2008a). In the flight bioassays, female *D. semiclausum* only discriminated at the

level of plant species; females preferred *B. juncea* over *B. nigra* plants, irrespective of *B. nigra* origin. Females of *C. glomerata* on the other hand, discriminated among the different *B. nigra* populations as well as between some *B. nigra* populations and the *B. juncea* cultivar. Chemical analysis of the headspace of *P. xylostella*-damaged plants revealed considerable differences among the various *Brassica* populations. Volatile blends emitted by the *B. nigra* populations mainly differed in terms of quantities of the various compounds, whereas the volatile blend of *B. juncea* also contained chemicals that were not emitted by *B. nigra*. Levels of volatile hydrolysis products of GS, i.e. allyl isothiocyanate, 3-butenyl isothiocyanate and 3-methyl-3-butenenitrile reflected the differences in GS composition between the two plant species.

Differences in behavioural preferences suggest that the two parasitoid species use different compounds or mixtures of compounds in the volatile blend as foraging cues. The two herbivore species may also differentially induce specific volatiles. Here we only analysed the headspace of *P. xylostella*-infested plants. Previous studies have demonstrated that volatiles emitted by plants from the same species damaged by different herbivores are very similar (Blaakmeer et al. 1994; Geervliet et al. 1997). Furthermore, other cues such as visual stimuli as well as volatile emissions produced by the host (Steinberg et al. 1993; Agelopoulos et al. 1995) could play an important role in the host-finding process and the final decision to alight on a plant (but see Bruinsma 2008). In addition, some parasitoid species including *C. glomerata* (Geervliet et al. 1998) modify their response to HIPV's through the process of associative learning (see Vet et al. 1995). Thus, learning by parasitoids could enhance the attraction to plant populations emitting specific volatile blends. However, learning capabilities in parasitoids are species specific and are expected to depend on the degree of dietary specialisation of the parasitoid and its host (Vet and Dicke 1992; Steidle and van Loon 2003). Nevertheless, selection on the production of parasitoid attractants may be compromised if different parasitoid species without oviposition experience use different compounds or mixtures of compounds as orientation cues. Thus, selection on certain chemicals or chemical blends may enhance the attraction of one parasitoid species, but reduce the attraction of another. It is also possible that in different geographical regions the relative abundance of parasitoid-host interactions determines the direction of selection. In addition, naïve (=without oviposition experience) parasitoids may be adapted to respond to their local plant populations.

Several studies have reported significant intraspecific geographic variation in the expression of direct plant defences (Zangerl and Berenbaum 2005; Gols et al. 2008c). It is expected that this variation is the consequence of a range of biotic and

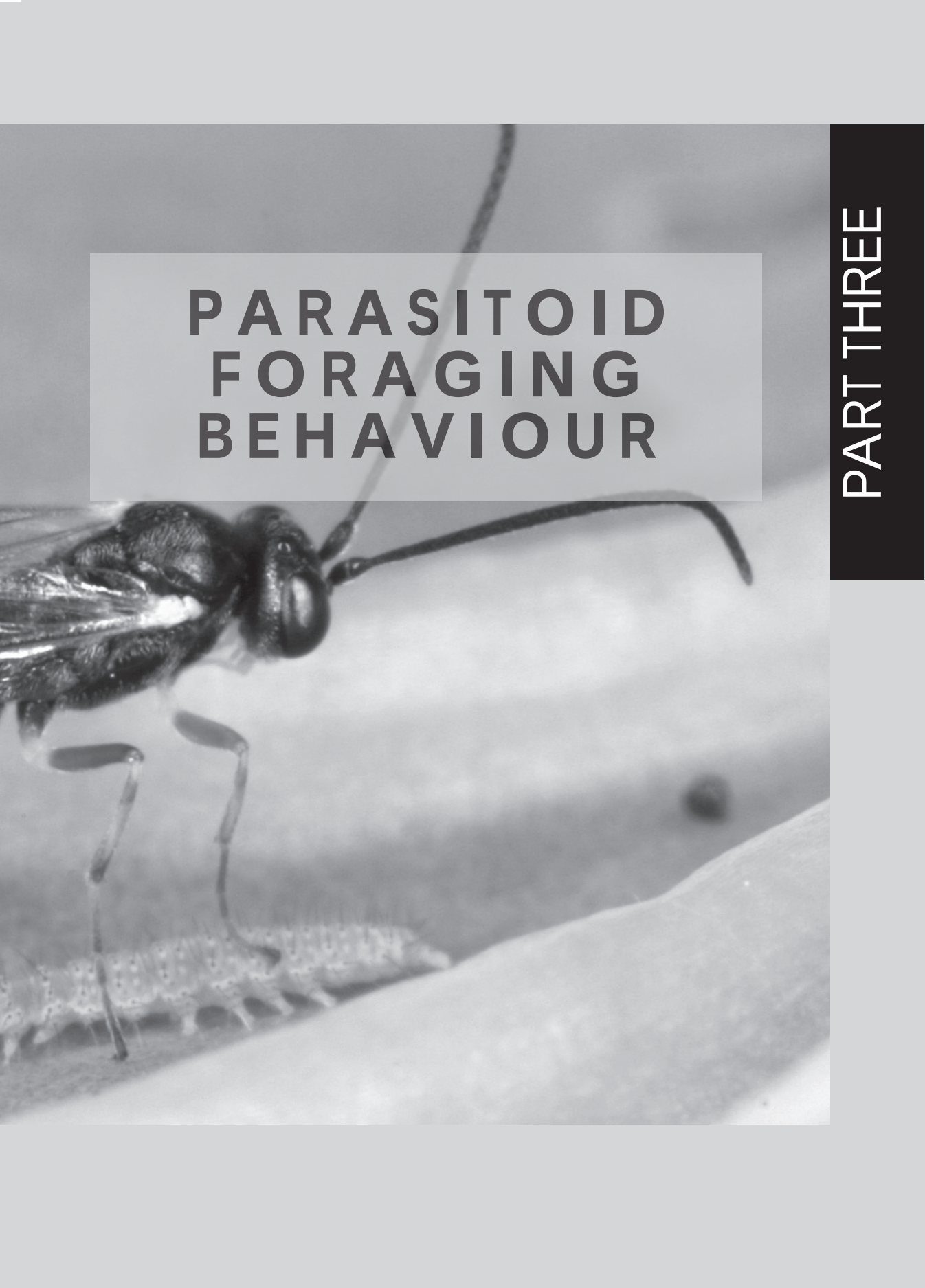
abiotic selection pressures mediated from the 'top-down' and 'bottom-up' selection forces that operate over variable spatial and temporal scales. Intraspecific variation in the expression of indirect defences, i.e. the production of volatile attractants, has also been reported and natural enemies are able to discriminate among volatile blends emitted by different cultivars of the same plant species (Loughrin et al. 1995; Geervliet et al. 1997; Gouinguene et al. 2001; Krips et al. 2001). Cultivars, however, are the result of artificial selection and consequently their volatile blends may have changed compared to those emitted by plants that have evolved under natural selection pressures. Little is known about the effects of local natural selection pressures on the production of volatiles and how these might affect the behaviour of natural enemies.

In summary, this study has examined and compared both indirect and direct defences in two wild *B. nigra* populations that probably have been exposed to different selection pressures, as well as in two mustard cultivars. The mustard populations differed quantitatively in the expression of GS and this was more pronounced when the *B. nigra* populations were compared with the *B. juncea* population. However, differences in GS concentrations among the populations did not reflect the performance of the two different herbivore-parasitoids systems. The fact that we used insects that are highly specialised on GS containing plants could explain the lack of correlation between GS levels and herbivore/parasitoid performance. The mustard populations also differed in the expression of indirect defences. Differences in the production of HIPV's were mainly found between *B. nigra* and *B. juncea*, which was reflected in flight behaviour of *D. semiclausum*, but not in that of *C. glomerata*. In nature, plants are not only attacked by insects but also by other antagonists including pathogens and mammalian herbivores. Plant populations should therefore exhibit frequency-dependent selection to a range of attackers that most commonly occur in their locality, and evolve mechanisms to reduce their most damaging and/or predictable enemies. Trade-offs between different defence traits, which are shaped by natural selection, determine which genotypes are present in a population, and depend on the relative strength of interactions between the plant, its attacker and natural enemies up the food chain. Future studies should aim at shedding more light on the behavioural and/or physiological mechanisms that mitigate conflicts between direct and indirect defence strategies in plants.

Acknowledgements

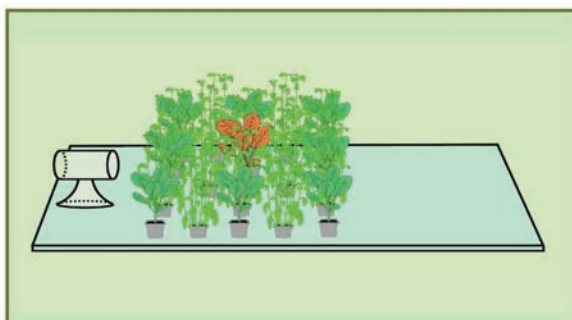
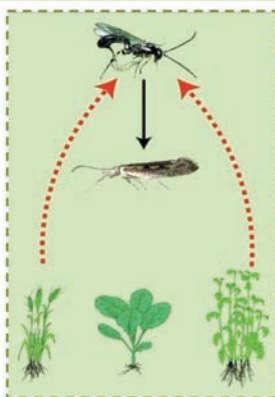
The authors would like to thank Leo Koopman and André Gidding for rearing the insects and Unifarm, especially Bert Esssenstam for rearing the plants.





PARASITOID FORAGING BEHAVIOUR

PART THREE



8

Reduced foraging efficiency of a parasitoid under habitat complexity: implications for population stability and species coexistence

by R. Gols, T. Bukovinszky, L. Hemerik, J.A. Harvey,
J.C. van Lenteren & L.E.M. Vet

Published in *Journal of Animal Ecology* 74: 1059-1068 (2005)

Habitat complexity may stabilise interactions among species of different trophic levels by providing refuges to organisms of lower trophic levels. Searching behaviour of the parasitoid, *Diadegma semiclausum*, was followed in different semi-field set-ups, a low and high-density monoculture of *Brassica oleracea* and two intercrops, *B. oleracea* with *Sinapis alba* (also a member of the Brassicaceae) and *B. oleracea* with *Hordeum vulgare* (Poaceae). When a low-density monocrop of *B. oleracea* was compared with a high-density monocrop, no differences were found in the ability of the female wasps to locate a host-infested plant, *B. oleracea*, infested with *Plutella xylostella* that was placed in the centre of the set-up. The efficiency of the parasitoid to locate the host-infested plant was differentially affected by the species composition of the vegetation. Wasps entered the *Sinapis-Brassica* set-up faster, but took more time to find the host-infested plant than in the *Hordeum-Brassica* set-up. The horizontal arrangement, i.e., by mixing *S. alba* or *H. vulgare* with, or placing them as rows between *B. oleracea*, did not affect host-finding efficiency. Plant height did influence host finding. Wasps found the host-infested plants earlier in the set-up with short *Sinapis* plants compared with tall *Sinapis* plants. Once the wasps had landed on the host-infested plant, the surrounding vegetation did not affect time needed to parasitise five consecutive hosts on the same infested plant, regardless of the composition or horizontal/vertical arrangement of the set-up. Chemical and structural refuges in complex landscapes may play an important role in the persistence of this system through dampening oscillations of parasitoid and host populations.

Key-words: Brassicaceae, *Diadegma semiclausum*, *Plutella xylostella*, proportional hazards model, refuges

Introduction

One of the biggest challenges in contemporary ecology is to understand better the factors that confer stability to communities and ecosystems (1973; McCann et al. 1998). The importance of habitat heterogeneity and refuges in stabilising the dynamics of tightly coupled predator-prey interactions has been empirically demonstrated (Huffaker 1958; Huffaker et al. 1963; Ellner et al. 2001). Furthermore, Gauld, Gaston & Janzen (1992) have also speculated that the spatial complexity of plant communities in the tropics may at least partially account for reduced species richness of parasitoids in these biomes. Parasitoid wasps are often specialised on one or a few host species and are therefore expected to have evolved intricate mechanisms to locate hosts in heterogeneous landscapes. In simple landscapes, such as crop monocultures, parasitoids frequently overexploit host populations, leading to boom-and-bust cycles that are clearly unstable and lead to high rates of local extinction (Hawkins 1994). This may be because in simple communities parasitoids are confronted with much less of a challenge to find hosts than is the case in more complex, dynamic communities and landscapes. Monocultures and intercrops of crop plants may therefore serve as a starting point to study the effect of landscape complexity on a suite of multitrophic interactions.

Insect parasitoids have to search for their hosts in spatially and temporally heterogeneous environments. As host encounter is directly correlated with the production of offspring, natural selection is expected to act strongly on the efficiency of parasitoids to find hosts. The success of parasitism involves several hierarchical steps such as habitat location, host location, host acceptance, host suitability and host regulation (Vinson 1976; Vinson and Iwantsch 1980a, b). Parasitoids are directed to their hosts through series of physical and chemical cues (reviewed by Godfray 1994; Quicke 1997), thereby gradually restricting the area in which the host can be found. Chemical cues associated with the host or the host microhabitat have been shown to play an important role in host plant and host location (for reviews see Vinson 1991; Tumlinson et al. 1992; Vet and Dicke 1992; Takabayashi and Dicke 1996; Dicke 1999b;).

Price *et al.* (1980) emphasised the importance of plants and their effect on interactions between insect herbivores and their natural enemies. However, the role of vegetation characteristics on enemy attraction and arrestment in the habitat has received little attention (Sheehan 1986). Plant structural characteristics such as geometry and size of plants as well as variation in the connectivity of stems or leaves may influence foraging success of a parasitoid (Andow and Prokrym 1990; Gingras et al. 2002). In the present study, we have investigated whether the structure

and the composition of a habitat influence parasitoid foraging behaviour. Comparison of searching behaviour of individual parasitoids in habitats differing in plant density, plant species composition and spatial arrangement may give more insight of how vegetation characteristics affect host-finding efficiency of parasitoids.

We studied host-location behaviour of *Diadegma semiclausum* Hellén (Hymenoptera, Ichneumonidae, Campopleginae) in a semi-field set-up in a monocrop and two intercrops in different spatial arrangements. *Diadegma semiclausum* is a solitary endoparasitoid that is specialized on the diamondback moth *Plutella xylostella* L. (Lepidoptera, Plutellidae), which is itself a specialist herbivore on plants in the Brassicaceae. *Diadegma semiclausum* uses herbivore-induced plant volatiles to locate host-infested plants (Wang and Keller 2002; Ohara et al. 2003b) and can attack and successfully develop in all four larval stages of *P. xylostella* (Yang et al. 1993). The aim of the present study was to determine whether composition and structural characteristics of a habitat affect the tendency of a parasitoid to initiate foraging and to find a host-infested plant. In addition, we studied whether plants surrounding the host-infested plant affect oviposition rates once the parasitoid has found a host infested plant.

Materials and methods

Insects

Diadegma semiclausum was collected from Brussels sprout fields in the vicinity of Wageningen (The Netherlands) and was reared on Brussels sprout plants infested with larvae of the diamondback moth. Sprout plants infested with the host, were obtained from a mass rearing maintained at the laboratory at 20 ± 2 °C, a relative humidity of 70% and a L16:D8 photoperiod. Parasitoid cocoons were collected weekly and were transferred to a clean insect cage (37 x 40 x 30cm) without plants. To obtain mated females, seven females and seven males that were less than 24 h old, were transferred to a glass tube (20 cm long, 5 cm in diameter) covered with fine meshed gauze at both ends. One day before an experiment, females were separated from the males and were transferred to a new cage (42 x 22 x 22cm). Wasps were provided *ad libitum* with water and honey, which was administered to the gauze. For the observations we used females without oviposition experience that were 5-10 days old. The rearing was maintained in a climate room, L16:D8 photoperiod at 20 ± 2 °C and a relative humidity of 70%.

Plants

The crucifer Brussels sprout (*Brassica oleracea* L. gemmifera cv. Cyrus) served as a host plant for *P. xylostella*. To investigate how a related uninfested plant species may interfere with parasitoid foraging behaviour, white mustard *Sinapis alba* L. cv. Carnaval was chosen as a cruciferous companion plant. Although mustard is a suitable host plant for *P. xylostella*, it was not infested with *Plutella* in the current study. To study the effect of a phylogenetically unrelated species (Poaceae), malting barley *Hordeum vulgare* L. cv. Video was mixed with Brussels sprout. All plants were grown in a greenhouse compartment at 20-28 °C, 40-80% r.h. and L16:L8 photoperiod. To obtain similar amounts of above-ground biomass for mustard and barley (20-25 g), the number of plants per pot was varied; nine plants per pot for mustard and 18 plants per pot for barley. Barley and mustard plants were 4-5 weeks old when they were used in experiments. Brussels sprout plants (one plant per pot) were used when they were 7-8 weeks and had attained 25 to 30 g above-ground biomass.

Experimental set-up

The effect of plant density, plant species composition and spatial arrangement of the plants on the searching behaviour of female *D. semiclausum* was studied in three different experimental set-ups (see below). All experiments were conducted in a greenhouse compartment in a tent-like construction (330 x 175 x 280 cm), which was made out of white sheets to obtain a contrasting background (for details see Wiskerke and Vet 1994). The greenhouse compartment was continuously ventilated, which created a turbulent air-current inside the tent. The climatic conditions were the same as mentioned in the *Plants* section, but without additional illumination. Plants were arranged on a table that was placed inside the tent. The basic set-up consisted of nine Brussels sprout plants in three rows of three plants with only the central plant containing hosts (target plant). Fifteen third instar *P. xylostella* were evenly distributed over three leaves in the late afternoon of the day prior to the experiments. These densities coincided with moderately high densities found in local Brussels sprout fields (T. Bukovinszky, personal observation). In the experiments, female wasps were observed continuously until they had located the target plant and had parasitised five out of the 15 *Plutella* larvae. The observations were conducted between 10.00 and 16.00 h. Experiment I and II were carried out from May to September 2002 and experiment III was carried out from September to November 2002. Within these two experimental periods different set-ups were tested randomly and at least two treatments were tested on the same day.

Experiment I: effect of plant density

In this experiment, searching behaviour of *D. semiclausum* in

a low plant density set-up (nine plants) was compared with its searching behaviour in a high-density (9+12 plants) monotypic set-up with Brussels sprout (Fig. 1A).

Experiment II: effect of plant species composition and plant arrangement

In experiment II, a second plant species was added by replacing 12 sprout plants from the high-density set-up with either barley or mustard. At the same time, the horizontal arrangement of the plants was varied. Brussels sprout plants were interspersed with either twelve pots of mustard or barley (mixed configuration, Fig. 1A). In the row configuration (Fig. 1B), mustard or barley were arranged as two rows of six plants between the sprouts. The resulting data sets were analysed together with the high-density sprouts data set obtained in experiment I.

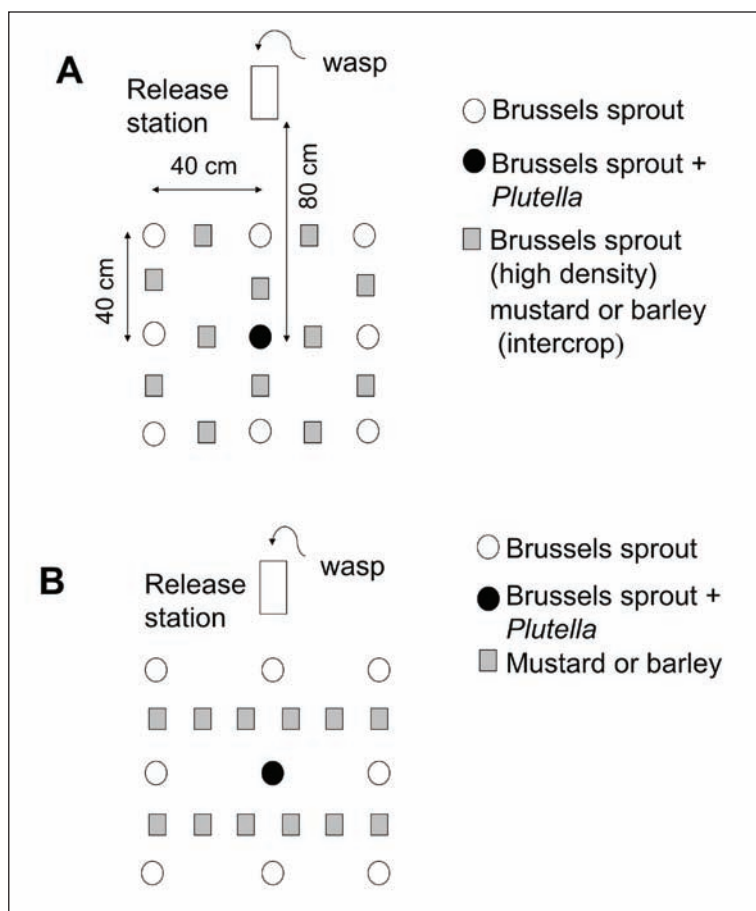
Experiment III: effect of plant height

In Experiment II, the mustard plants were taller than the Brussels sprout plants. In this set-up, we investigated the effect of the relative height of mustard and sprout plants on host location behaviour of the parasitoid. Sprout with mustard plants were positioned in the row-configuration (Fig. 1B) in three different vertical arrangements. Brussels sprout plants were either arranged with short (3 weeks old) mustard plants or tall (4 weeks old) mustard plants. In the third set-up, Brussels sprout plants, including the host-infested plant, were elevated by placing them on 25 cm high platforms and mixed with tall (4 week old) mustard plants. In the latter set-up sprout plants reached the same height as the tall mustard plants.

Direct observations

The behaviour of naïve *D. semiclausum* females was observed continuously and recorded using a handheld microcomputer (Psion Workabout Psion PLC) and 'The Observer' software 3.0 (Noldus Information Technology 1993). To enhance the motivation of the parasitoids to forage, a female was put in a glass vial with a leaf disc taken from a damaged Brussels sprout plant from which the larvae and their products had been removed. The vial with the parasitoid was placed in a release station, a glass cylinder (30 cm long, diameter of 15 cm) placed on a 10 cm high socket, which was placed at one end of the table (Fig. 1), 80 cm from the target plant. The observation started when the wasp left the release station and ended with the fifth oviposition. Four behavioural states were observed, i.e., flying, searching (includes walking, probing with the ovipositor and antennation), standing still and preening. Every time the wasp inserted the ovipositor into a host, it was recorded as an oviposition. Searching, standing still and preening could either occur on a Brussels sprout plant infested with hosts, sprout plants without hosts or on the companion

Figure 1: Distribution of plants in the experimental set-up for the behavioural observations in the flight chamber in mixed (A) and row (B) configuration. A female wasp is set free from the release station and her behaviour is recorded until she has achieved five ovipositions on the host-infested plant (Brussels sprout + *Plutella*).



plant, barley or mustard. Time spent outside the set-up (table and tent fabric), which was recorded as well, was excluded before the data files were analysed. Wasps that flew to the ceiling right after release and did not fly into the system within five minutes, as well as wasps that left the system before making five successive ovipositions (15 out of 244 observations) were excluded from the data analysis. More than 60% of the released parasitoids succeeded to parasitise five hosts. At least twenty-five observations were obtained for each set-up. Each wasp was used only once and plants infested with *P. xylostella* were used one to five times. Parasitised *Plutella* larvae were replaced by unparasitised ones after each observation.

Description of the model and analysis of the data

Cox's proportional hazard model is a stochastic model, which assumes that beginning or ending a behavioural element has a stochastic component. The model is formulated in terms of the hazard rate. This hazard rate is the probability per time unit that a specific event will occur, e.g., first landing on the vegetation or

landing on a host-infested plant. It is assumed that the parasitoid has a basic tendency to perform a behavioural element, the baseline hazard. The observed hazard rate is assumed to be the product of the baseline hazard and the multiplicative effects of fixed variables or so-called covariates (eqn 1).

$$h(t, z) = h_0(t) \exp\left(\sum_{i=1}^p \beta_i z_i\right) \quad \text{eqn 1}$$

where $h(t; z)$ is the probability per second that an event will occur, $h_0(t)$ is the baseline hazard and z_i are the fixed covariates that potentially influence tendencies to perform a certain behaviour. The β_i coefficients, which are estimated in the analysis, denote the relative strength of the effect of each covariate; i.e. $\exp(\beta) = 1$, there is no effect of the covariate, and $\exp(\beta) < 1$ (> 1), the covariate decreases (increases) the hazard rate.

We selected plant density, plant composition and plant arrangement as factors in the habitat that may affect foraging behaviour of a parasitoid. The relative influence of these covariates is determined by means of partial likelihood maximisation (Kalbfleisch and Prentice 1980). For a more detailed description of the model we refer to Haccou & Hemerik (1985), Hemerik & Driessen (1993), and Kalbfleisch & Prentice (1980).

Above, the covariates are assumed to be fixed, i.e. time-independent. However, visits to non-target plants and companion plants during exploration of the set-up are likely to influence the tendency to arrive at the target plant. Therefore, the model was extended with a time-dependent covariate $v(t)$. This time-dependent covariate $v(t)$ takes into account the visits to the nonhost plants as a percentage of the visits to all plants without hosts until it arrives at the host plant and is expressed as follows:

$$v(t) = 100 \frac{c(t)}{c(t) + n(t)} \quad \text{eqn 2}$$

where $c(t)$ is the number of visits to nonhost plants (barley or mustard) and $n(t)$ is the number of visits to noninfested Brussels sprout plants between time 0 and t . The general form of the extended proportional hazards model with fixed and time-dependent covariates is described in eqn 3:

$$h(t; z, v(t)) = h_0(t) \exp\left(\sum_{i=1}^p \beta_i z_i + \sum_{j=1}^q \delta_j v_j(t)\right) \quad \text{eqn 3}$$

where it is assumed that the hazard rate $h(t; z, v(t))$ at time t has a time-dependent and a time-independent component, $v_j(t)$ and z_i ($j = 1, \dots, q$; $i = 1, \dots, p$) respectively, where δ_j and β_i give the relative strength of the covariates $v_j(t)$ and z_i .

A summary of the covariates used in the three experiments and their coding is presented in Table 1. In the three experiments, plant density (low, high), composition (monotypic, mustard and

Table 1: Coding of the different covariates in the three experiments

barley intercrop) configuration (mix, row) and height (small, tall, elevated) were treated as fixed covariates. These covariates were handled as categorical variables and were coded 0 or 1. The time-dependent covariate was expressed as described in eqn 2.

Experiment I: plant density	
1. Is Brussels sprout density low?	Yes/No = 1/0
Experiment II: plant species composition and plant arrangement	
2. Is the companion plant barley?	Yes/No = 1/0
3. Is the companion plant mustard?	Yes/No = 1/0
4. Are plants arranged in the row configuration?	Yes/No = 1/0
5. Percentage of visits to companion plants	$c(t) * 100/(c(t)+n(t))$ ¹
Experiment III: plant height	
6. Are the mustard plants tall?	Yes/No = 1/0
7. Are the Brussels sprout plants elevated?	Yes/No = 1/0
8. Percentage of visits to companion plants	$c(t) * 100/(c(t)+n(t))$ ¹

¹ $c(t)$ is the cumulative number of visits to non-host plants and $n(t)$ is the cumulative number of visits to non-infested *B. oleracea* plants

We considered as the events of interest arrival at the first plant in the set-up, arrival at the target plant and host encounter on the target plant. These events refer to different phases of the host selection behaviour of a parasitoid, respectively, habitat selection, host plant selection and host selection (Vinson 1976). Therefore, the data set was split into different parts, i.e., from leaving the release platform until arrival at the first plant in the set-up, from leaving the release platform until arrival at the host-infested plant. For oviposition tendencies, the time from landing on the target plant until first oviposition and the times between successive ovipositions were used. In the analysis, the intervals between the five successive ovipositions were coded by categorical covariates with the interval from landing on the target plant until first oviposition as baseline. The effect of the appropriate covariates, i.e., the fixed covariates from Table 2, on the tendency to oviposit was investigated for all three experiments. For the comparison of first landing in the set-up and time to accomplish five successive ovipositions, the model with fixed covariates (eqn 1) was used. For comparison of arrival tendencies at the host plant the covariate 'visits to companion plants' was included and the extended model was used (eqn 3).

Results

Effect of plant density (experiment I)

Increasing the number of conspecific plants in the set-up from nine to 21, did not significantly influence arrival times at infested plants (Fig. 2, Table 2).

Effect of plant species composition (experiment II)

To investigate whether the differences in set-ups influenced the motivation of the parasitoid to enter a set-up, arrival tendencies at any plant since flight initiation were studied first. Replacing Brussels sprout plants with heterospecifics (mustard or barley) influenced searching parasitoids in different ways. Compared with the high-density sprout monocrop, wasps tended to land

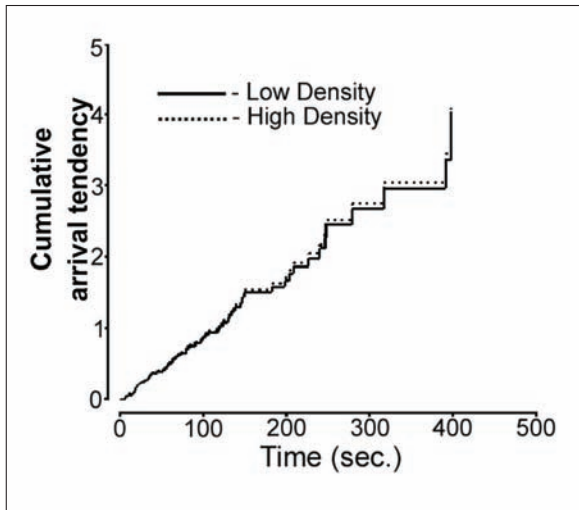


Figure 2: Cumulative tendency (or hazard rate) of female *D. semiclausum* to arrive at the host-infested plant in a low and high density Brussels sprout monocrop. The survivor function $S(t) = \exp(-\int_0^t h(s, z) ds)$ describes the proportion of individuals still at risk at time t . Therefore a value of the cumulative hazard rate at time t ($\int_0^t h(s, z) ds$) of say 2 means that 13.5% of the individuals is still at risk, i.e. has not arrived at the host-infested plant.

earlier in the mustard intercrop and later in the barley intercrop (Fig. 3A, Table 2). However the difference in arrival tendency was only significant between mustard and barley intercrops. Wasps flew into the set-up significantly earlier in the mustard than in the barley intercrop ($\chi^2 = 9.29$, d.f. = 3, $P = 0.03$).

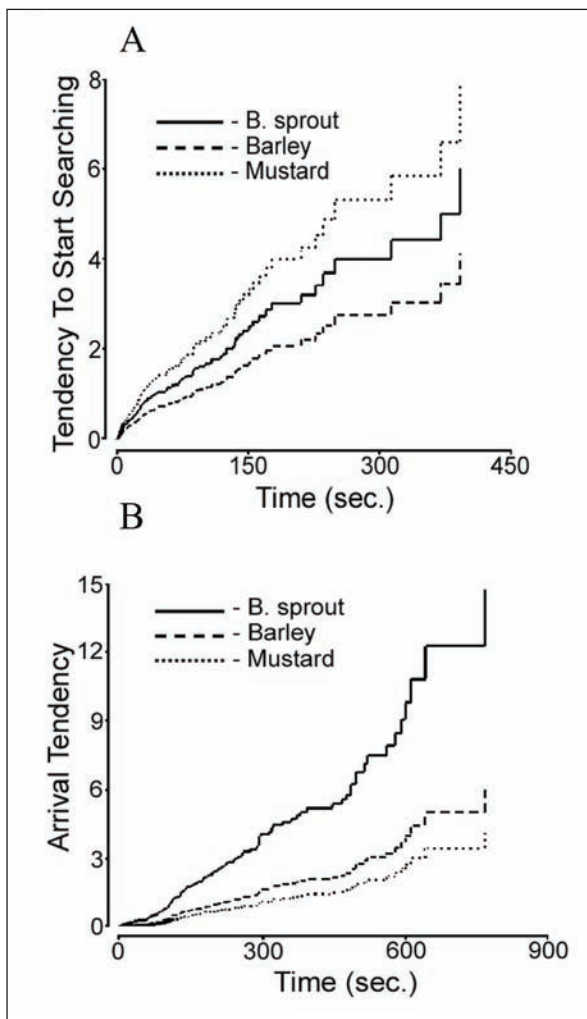
Comparison of arrival tendencies at the host-infested plant in the different set-ups showed that wasps arrived significantly later at the target plant in the mustard and barley intercrop than in the sprout monocrop (Fig. 3B, Table 2). The lowest arrival tendencies were observed for the mustard intercrop. The covariate that accounts for visits to companion plants had a significant effect on the hazard rate to arrive at the target plant (Table 2). An increase in the proportion of landings on companion plants increased the probability of arrival at the target plant. To investigate whether visits to nonhost plants depended on intercrop plant species, it was tested whether there was an interaction between the covariate for plant species and the one accounting for visits to companion plants. The data from experiment II were analysed with an extra time-dependent covariate, the product of the covariate for plant species and visits to companion plants. The effects on the arrival tendency of the interaction, i.e., the product of covariates 2 and 5 (Table 1) for barley and the product of covariate 3 and 5 for mustard were analysed separately for the two set-ups. The effect of percentage of visits to barley in the barley intercrop was not significant anymore ($\chi^2 = 2.79$, d.f. = 5, P

= 0.73), whereas for the mustard intercrop percentage of visits to companion plant still had a significant effect on arrival tendency ($\chi^2 = 12.46$, d.f. = 5, $P = 0.03$).

Effect of plant arrangement (experiment II)

Positioning of plants in a mixed or row arrangement neither influenced motivation of the parasitoids to fly into the set-up, nor their arrival tendencies at the host plant (Table 2).

Figure 3: Cumulative tendency (or hazard rate) of female *D. semiclausum* to land at any plant in the set-up after being released outside the set-up (A) and to arrive at the host-infested plant in the centre of the set-up (B). Set-ups consist of Brussels sprout (high density), barley-sprout intercrop or mustard-sprout intercrop in the mixed configuration.



Effect of plant height (experiment III)

Wasps arrived at the target plants significantly later when sprout plants were surrounded by tall mustard than when sprout plants were surrounded by short mustard plants (Fig. 4, Table 2). Elevating the sprout plants to the same height as the tall mustard

plants resulted in arrival tendencies similar to the tall mustard set-up. An increasing percentage of visits to non-host plants significantly increased the tendency to arrive at the host-infested plant (Table 2).

Covariates	χ^2 (d.f.)	Estimated effect of Exp β
Experiment I: arrival tendencies at host-infested plant		
Plant density	0.01(1) NS	0.974
Experiment IIa: first landing tendencies in the set-up		
Companion plant barley	1.10(3) NS	0.754
Companion plant mustard	1.86(3) NS	1.447
Configuration (mix/row)	0.70(3) NS	0.843
Experiment IIb: arrival tendencies at host-infested plant		
Companion plant barley	12.99(4) **	0.332
Companion plant mustard	28.11(4) **	0.120
Configuration (mix/row)	1.67(4) NS	0.763
Visits to companion plant	14.28(4) **	1.012
Experiment III: arrival tendencies at host-infested plant		
Tall mustard	23.39(3) *	0.182
Elevated Brussels sprout	24.65(3) *	0.176
Visits to companion plant	10.34(3) *	1.1013

NS: not significant; *: $P < 0.05$; **: $P < 0.01$

Table 2: Estimated effects of the covariates in the different experiments

Host encounter rates

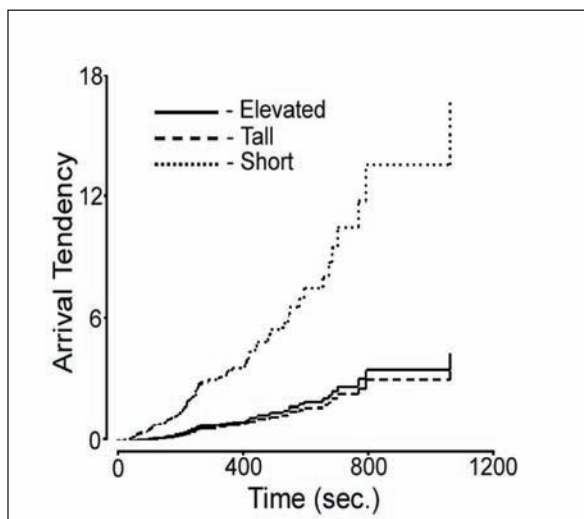
After arriving at the target plant, the tendency to accomplish five ovipositions was not affected by the number of previous ovipositions in any of the three experiments. The oviposition tendency was neither influenced by plant density ($\chi^2 = 0.03$, d.f. = 5, $P = 0.99$), nor by replacing sprouts by barley ($\chi^2 = 1.26$, d.f. = 7, $P = 0.99$) or by mustard ($\chi^2 = 1.94$, d.f. = 7, $P = 0.96$). There was also no effect of horizontal arrangement (row vs. mixed configuration, $\chi^2 = 8.81$, d.f. = 7, $P = 0.27$) nor of vertical arrangement of the plants (short vs. tall mustard, $\chi^2 = 4.56$, d.f. = 6, $P = 0.6$ and short mustard vs. elevated sprouts/tall mustard $\chi^2 = 5.66$, d.f. = 6, $P = 0.46$).

Foraging behaviour of *Diadegma semiclausum*

Females patrolled the habitat at close range (about 10 cm). When the female wasp had located the host plant, she tended to land only on the host-infested leaves. She explored the sites of feeding damage although *Plutella* larvae tend to move away from their feeding sites. Sometimes, the wasps explored already

visited sites again. Occasionally, a female attacked a host it had already parasitised. Time allocation of *D. semiclausum* females to the different behavioural elements in the different set-ups

Figure 4: Cumulative tendency (or hazard rate) of female *D. semiclausum* to arrive at the host-infested plant in a mustard-sprout intercrop in the row configuration with varying heights of sprout plants relative to the height of mustard. The set-up consisted of either short mustard, tall mustard or elevated sprout plants with tall mustard plants.



is presented in Table 3. When time allocation in low and high density Brussels sprout set-ups (experiment I) is compared, no significant differences were found, except for flying. Wasps in the high-density sprout monocrop spent more time flying than in the low-density monocrop. In experiment II, horizontal configuration and plant composition were compared. Plant species only affected searching on companion plants. Female wasps were searching longer on mustard than on barley. The mixed configuration reduced the time spent preening on infested and searching on uninfested host-plants and decreased the total time to accomplish five ovipositions. The height of Brussels sprout relative to mustard (experiment III) influenced foraging on infested host-plants and companion plants and flying (Table 3: Experiment III). Time allocation was not significantly different in the tall mustard and the elevated sprout set-up. However, in the short mustard set-up wasps foraged significantly less on mustard plants and spent significantly less time flying compared to the other two set-ups in experiment III.

Table 3: Time (in s) allocated to different behavioural elements (mean $\pm$ SE) based on direct observations of foraging behaviour of *D. semiclausum* in the three different experiments as described in the Materials and Methods section.

Behavioural elements	Infested host plant			Noninfested host plant Total	Companion plant			Fly	Total
	Search	Preen	Stand still		Search	Preen	Stand still		
Experiment I: plant density									
Sprout low density (<i>n</i> =37)	302±21	137±14	64±21	34±11				123±11	663±47
Sprout high density (<i>n</i> =26)	299±28	150±17	48±14	37±9.1				190±31	723±64
Mann-Whitney <i>U</i> -test	NS	NS	NS	NS				0.01	NS
Experiment II: plant species composition and plant arrangement									
Barley mix (<i>n</i> =25)	273±22	103±8.5	32±15	7.6±2.7	23±7.8	8.2±2.3	8.0±4.3	237±28	692±53
Barley row (<i>n</i> =25)	339±38	148±17	70±23	36±11	21±5.9	10.8±3.2	10.1±5.5	284±30	918±78
Mustard mix (<i>n</i> =25)	255±21	103±12	31±12	7.3±3.5	86±16	4.3±1.8	5.9±2.5	280±30	777±56
Mustard row (<i>n</i> =25)	321±31	136±15	65±22	12.2±3.6	94±17	6.1±2.1	7.4±3.6	336±33	977±78
Scheirer-Ray-Hare extension of the Kruskal-Wallis test									
Configuration	NS	0.04	NS	0.02	NS	NS	NS	NS	0.01
Species	NS	NS	NS	NS	<0.001	NS	NS	NS	NS
Species*configuration	NS	NS	NS	NS	NS	NS	NS	NS	NS
Experiment III: plant height									
Mustard tall (<i>n</i> =25)	279±27	132±16	15±4.4	23±8.1	132±29	9.5±2.1	5.0±1.8	342±42	938±79
	AB	AB			A	A		A	A
Sprout elevated (<i>n</i> =25)	346±26	182±16	24±8.5	24±10.2	126±16	11.9±2.7	4.3±1.4	374±34	1091±74
	A	A			A	A		A	A
Mustard short (<i>n</i> =25)	247±25	118±9.9	6.4±2.3	11±3.6	21±5.6	2.1±0.8	1.8±0.8	172±16	579±35
	B	B			B	B		B	B
Kruskall-Wallis test	0.02	0.01	NS	NS	<0.001	0.001	0.04	<0.001	<0.001

In each experiment levels of significance are given, NS: not significant.

In experiment III, multiple comparisons were conducted based on rank numbers. The critical value takes into account the total number of comparisons. Different letters within one column denote set-ups with significantly different means ($P<0.05$).

Discussion

A parasitoid is usually directed to its host through a progressive reduction of searching area by means of physical and chemical cues (Vinson 1976). The present study demonstrated that the composition and structure of the vegetation affected host-finding efficiency of female *D. semiclausum*. As females were not influenced by the density of Brussels sprout in a monocrop set-up, the differences in foraging behaviour in the intercrop set-up were due to species-related effects and not simply because of changes in biomass or in leaf area of plants. Although in both

intercrop combinations the time to locate a host-infested plant was longer than in the monocrop, different patterns were observed. Replacing some of the Brussels sprout plants with another crucifer increased tendencies to fly into and land in the habitat for the first time compared to the monocrop, whereas replacing some sprout plants by the cereal barley decreased the tendency of the parasitoid to enter the vegetation. Apparently, cues that initiate flying towards the barley intercrop were less strong than in the mustard intercrop. Bukovinszky *et al.* (2005) have found that volatiles emitted by barley are less attractive to *D. semiclausum* than volatiles emitted by mustard or sprouts. Once the parasitoid had entered the set-up, both intercrops decreased tendencies of *D. semiclausum* to arrive at the host-infested plant.

Foraging behaviour of *D. semiclausum* is characterised by flying from one plant to another and searching the leaf surface of the visited plants (personal observation). In earlier studies, it was shown that *D. semiclausum* females fly upwind towards infested host plants indicating that volatile cues play a role in host plant location (Wang and Keller 2002; Ohara *et al.* 2003b), but visual cues may play a role as well. *Diadegma semiclausum* was clearly attracted to mustard even though there were no hosts present on this plant. The wasps appeared to land on the target plant only when this plant was encountered accidentally while flying from one mustard plant to another. Mustard and Brussels sprout both belong to the family of Brassicaceae and members of this family differ in glucosinolate composition and concentration (Reed *et al.* 1989; Fahey *et al.* 2001; Harvey *et al.* 2003). Members of the Brassicaceae also differ in their attractiveness to parasitoids (Benrey *et al.* 1998). Moreover, various cultivars of *B. oleracea* have been shown to differ in volatile emissions and differentially attract the parasitoid *C. glomerata* (Geervliet *et al.* 1996, 1997). *Diadegma semiclausum*, being a specialist on *P. xylostella*, prefers cruciferous plants. This parasitoid may have evolved an innate response to cues related to Brassicaceae and differences in chemical and physical characteristics between members of this family may determine the relative strength of these cues for the parasitoid (Vet *et al.* 1990). It has been demonstrated that *D. semiclausum* prefers odours from mustard over Brussels sprout as shown in olfactometer experiments (Bukovinszky *et al.* 2005). Differences in host quality mediated through the host plant may also affect parasitoid preferences for host plants (Fox and Eisenbach 1992). In other words, *D. semiclausum* may prefer to search a host plant on which the host performs best. Barley on the other hand, is an unrelated cereal species and may affect parasitoid foraging behaviour either through confusing effects of differences in odour-plume composition or by imposing a physical barrier hampering parasitoid movement.

Vegetation characteristics such as size, density and geometry may interfere directly with the movement of parasitoids

depending on the spatial scale at which foraging occurs (e.g. Andow and Prokrym 1990; Coll and Bottrell 1996; Idris and Grafius 2001). The present study shows that increasing plant density of conspecifics and placing heterospecifics in different horizontal arrangements does not influence foraging success of *D. semiclausum*. Female wasps were seen patrolling the canopy of plants at a very close distance (about 10 cm). This may explain why a reduction in physical apparency, by changing the height of Brussels sprout relative to the height of mustard, did not affect the host finding efficiency of the parasitoid. However, we also studied parasitoid foraging in a set-up with younger mustard plants. In this case, *D. semiclausum* arrived at the host plants much faster than in the older and taller mustard set-up. The more condensed canopy of the younger plants seemed to impede parasitoid movement between the stems of the mustard plants and reduced the amount of time spent on mustard. Although the parasitoid also spent time foraging on short mustard plants, the effective leaf searching-area was reduced on these plants and accidental encounters with the target plants increased. It cannot be excluded that there are differences in the headspace of mustard plants of different age, which may differentially attract *D. semiclausum*. This wasp is a highly mobile parasitoid exploring the vegetation by flying and hovering around plants. Differences in the mobility and size of a parasitoid could determine at what spatial scale structural characteristics of the vegetation affect foraging behaviour.

Once the target plant was located, the composition or configuration of the surrounding vegetation did not influence parasitoid exploitation of the host-patch, i.e. the tendency to oviposit in successive hosts. In all three experiments, after arrival at the host infested plants, wasps mainly searched on or hovered around the target plant. When occasionally the parasitoid did make excursions further away from the target plant, it easily returned to the host plants without being affected by the other plants in the set-up. Time spent between consecutive ovipositions is highly variable and seems to be influenced by the distribution of the hosts on the plant. Diamondback moth larvae are known to move away from their feeding site and appear to be able not to reveal their presence due to cues emanating from these areas (Wang and Keller 2002). Once a parasitoid has arrived at the host plant, cues directly related to the host or its presence (faeces, silk, feeding damage) are assumed to become important and olfactory/physical aspects of plants in the neighbourhood become less important.

In this study parasitoid foraging behaviour was examined in a greenhouse compartment where the searching range of the parasitoid was limited by the boundaries of the set-up. In the field, the wasp can move around over a much larger area and may spend less time per unit of surface area. If more attractive plants

in the habitat are visited more frequently, less attractive plants with hosts will escape parasitism. Depending on the distribution of the host over the differentially attractive host plants, foraging efficiency and lifetime reproductive success may be affected negatively. Parasitoid foraging efficiency may be further reduced by the presence of non-hosts on the food plants, although it has been shown that specialist parasitoids and predators can discriminate between odours from plants infested with hosts and non-hosts (Shiojiri et al. 2001; de Boer et al. 2004)

We have only investigated the role of the surrounding vegetation on host finding and subsequent ovipositions in the same host patch. However, parasitoids are well known to learn from their foraging experiences (reviewed by Vet et al. 1995). Gaining experience while re-orienting in the habitat may improve foraging success with time. Different habitat characteristics may not only influence arrival times, but also leaving tendencies from the habitat (Bukovinszky 2004). The way parasitoids acquire experience in diverse multi-patch environments and its impact on foraging success merits further study.

In experimental field plots with Brussels sprout parasitism rates of *P. xylostella* by *D. semiclausum* reach over 90% in August (Bukovinszky 2004), indicating that parasitoids easily exploit hosts in simple agro ecosystems. Refuges play an important role in sustaining multitrophic interactions, and in particular associations involving strongly co-evolved species, by preventing over-exploitation of prey or hosts by their natural enemies (Murdoch et al. 1987; Hochberg and Holt 1995; Biere et al. 2002).

Several kinds of refuges have been described, including spatial (Begon et al. 1995; Schrag and Mittler 1996), structural (Biere et al. 2002) temporal (Murdoch et al. 1987), and chemical (Vos et al. 2001) that enable herbivores to escape from their natural enemies. Refuges are characterised by their protective properties that are inversely related to the predation risk of prey individuals. In a series of classical studies, it was demonstrated that increasing heterogeneity can promote persistence of herbivorous mite populations that are prone to extinction by a exploitative predatory mite (Huffaker 1958; Huffaker et al. 1963; Ellner et al. 2001). Our results show that by increasing habitat complexity, parasitoid foraging efficiency is reduced. This reduction is based on physical and chemical characteristics of the plants surrounding the host-infested plant, which hinder the parasitoid in flight, or impede access of the parasitoid to chemical cues that are used to locate hosts. This affords the host two kinds of refuges that may play an important role in the persistence of the system through dampening oscillations of parasitoid and prey populations.

In conclusion we have shown that habitat composition and structure differentially affect the various phases of the host

selection process. Chemical and structural differences of plant and local habitat characteristics determine whether the effect of the associated plant on host plant location for the parasitoid is positive or negative. Many studies investigating the role of infochemicals on herbivore-enemy interactions are focused on single species plant-herbivore-natural enemy interactions under highly controlled conditions (for a critical review see Hunter 2002). Our study demonstrates that, to understand multitrophic interactions in natural and agro-ecosystems, a holistic field or semi-field approach is required in order to evaluate the impact of cue complexes.

Acknowledgements

The authors would like to thank Wouter Tigges, André Gidding and Leo Koopman for rearing the insects. Peter Mayhew and Marcel Dicke are acknowledged for giving critical comments on the manuscript. This study is part of the program “Enhanced biodiversity” funded by the Earth and Life Sciences of Dutch Scientific Organisation (NWO-ALW, project number: 014-22.031).

9

General discussion

by R. Gols

Introduction

Plants have evolved several defence strategies to reduce or prevent insect herbivory. These defence strategies can be divided into direct and indirect defences. Direct defences affect development and behaviour of the herbivores. For example, morphological adaptations such as spines, hairs and glossy leaf surfaces may interfere with movement of the herbivores on the plants, whereas chemicals such as toxins and digestibility reducers may negatively affect herbivore growth and survival (Schoonhoven et al. 2005). Indirect defences refer to plant traits that promote herbivore exploitation by natural enemies such as parasitoids and predators. Plants can provide alternative food sources and shelter, and they can produce attractive volatiles, which may lure natural enemies to herbivore-infested plants (Dicke 1999b). In my thesis I focused on chemical plant defences, i.e., the production of allelochemicals (direct defence) and volatile organic compounds that are produced in response to herbivore damage and are attractive to the herbivore's natural enemies (indirect defence).

The Brassicaceae provide an excellent plant family to study chemical defences. All brassicaceous species biosynthesise glucosinolates (GS), (Fig. 1). Following tissue damage, GS undergo hydrolysis catalysed by myrosinase enzymes leading to the formation of a range of products (Mithen 2001b). The final structure of these chemicals depends on the ambient conditions and the activity of enzymes that can further modify the structure of these products (Kliebenstein et al. 2005; Halkier and Gershenzon 2006). GS have been demonstrated to play a role in defence against generalist herbivores (Chew 1988; Louda and Mole 1991; Mithen 2001a), whereas specialist herbivores have evolved efficient detoxification or excretion mechanisms to circumvent the toxic effects of GS (Ratzka et al. 2002; Wittstock and Gershenzon 2002). Moreover, GS can induce oviposition and stimulate feeding by crucifer specialists (Renwick 2002) and some herbivores even sequester GS from their food plant and use them as defence against their own attackers (Müller et al. 2001). However, high levels of GS may also negatively affect the performance of specialist herbivores (Li et al. 2000; Agrawal and

Kurashige 2003). In addition, toxic secondary plant compounds may not only affect the development of herbivores but also that of their parasitoids (Hunter 2003; Ode 2006). The effects of GS-containing food plants on parasitoids developing inside hosts feeding on these plants has thus far received little attention in the literature (Benrey et al. 1998; Harvey et al. 2003; Sznajder and Harvey 2003) and addressing this was one of the objectives of my thesis.

Artificial selection in the Brassicaceae has given rise to important crops such as cabbages (*Brassica oleracea*), oilseeds (e.g., *B. nigra*, *B. juncea*) and mustard condiments (e.g., *B. nigra*, *Sinapis alba*). Resistance to and persistence of pesticides in the environment have promoted the search for alternative methods to control pests in agricultural systems. Reproductive success in parasitoids is closely correlated with the female's efficiency in finding hosts; therefore, parasitoids have evolved efficient foraging strategies to locate hosts in heterogeneous environments. The role of herbivore-induced plant volatiles (HIPV) in host-finding behaviour of parasitoids that attack larval stages of insect herbivores is well studied (Dicke 1999a; Turlings et al. 2002; Heil 2008). *Brassica* cultivars, especially those of *B. oleracea*, have often been used with respect to investigating HIPV and their attractiveness to the herbivore's natural enemies (see Table 3 in Chapter 1). However, through artificial selection, domesticated plants often contain modified levels of primary and secondary metabolites when compared with their wild progenitors. This modified chemistry in cultivated plants may affect the performance and behaviour of insects associated with these plants. In this thesis, I used both cultivated and wild brassicaceous populations and compared the development of both herbivores and their parasitoids on these different populations (parts I and II). In addition, the attraction of parasitoids to HIPV was compared in different wild and cultivated brassicaceous plant species (part II).

Importantly, plant-mediated effects on parasitoid performance and behaviour can act negatively through the production of phytotoxins (direct defence) and positively through the production of volatile attractants (indirect defence). These two plant defence traits are not necessarily mutually exclusive (Havill and Raffa 2000; Hunter 2003). For instance, the plant hormone jasmonic acid plays an important role in the induction of both direct and indirect plant defences (Dicke and van Poecke 2002). In other words, a plant with high levels of direct defences may also have high levels of indirect defences and *vice versa*. A conflict may arise when a herbivore's enemy is attracted to plants that have a negative impact on its own development (Havill and Raffa 2000). Yet, plants may also be able to differentially control the

expression of their direct and indirect defences (Kahl et al. 2000) and cross talk between signaling pathways may prevent potentially opposing directions of plant defence strategies (De Vos et al. 2005). However, direct and indirect plant defences are usually studied independently (see chapter 1 for studies in the Brassicaceae) with a few exceptions (see e.g., Havill and Raffa 2000). This potential conflict between direct and indirect plant defence is the subject in part II.

The majority of studies investigating the attractiveness of HIPV to parasitoids are based on windtunnel or Y-tube olfactometer experiments (for a critical review see Hunter 2002). A few studies have demonstrated that modification of volatile emissions results in enhanced attraction of natural enemies in the field (Khan et al. 1997; De Moraes et al. 1998; Thaler 1999; Halitschke et al. 2008). However, these studies were conducted in monocultures of mainly crop plant species. Little is known about the spatial scale at which these plant volatiles are perceived by parasitoids in natural environments. In addition, in natural habitats parasitoids have to forage in heterogeneous environments consisting of many different plant species that may create structural and chemical barriers. Recently, some studies have challenged natural enemies of herbivores with slightly more complex volatile blends. For example, Shiojiri et al. (2001) investigated the attraction of two parasitoid species to plants that were simultaneously damaged by host and non-host caterpillar species. Dicke et al. (2003) investigated the response of a predatory mite to mixtures of volatile emissions produced by conspecific plants infested with host and non-host prey as well as mixtures of heterospecific plants. Both studies showed that some natural enemies are able to discriminate between more complex volatile blends. In part III of this thesis, I studied parasitoid foraging behaviour in a more complex setting. More specifically, I investigated the role of other cruciferous and non-cruciferous plants surrounding a host-infested plants as well as the role of structural variation in the habitat.

I used lepidopteran herbivores and their hymenopteran larval endoparasitoids with different food plant and host specialisation,

Parasitoid characteristics				Host characteristics		Chapter
Parasitoid species	Host breadth	Solitary/gregarious	Feeding strategy: tissue/haemolymph	Host species	Dietary breadth	
<i>Diadegma semiclausum</i>	specialist	solitary	tissue	<i>Plutella xylostella</i>	specialist	3, 4, 7, 8
<i>D. fenestrata</i>	generalist	solitary	tissue	<i>P. xylostella</i>	specialist	4
<i>Cotesia rubecula</i>	specialist	solitary	haemolymph	<i>Pieris rapae</i>	specialist	5
<i>C. glomerata</i>	specialist	gregarious	haemolymph	<i>P. brassicae</i>	specialist	6, 7
<i>Microplitis mediator</i>	specialist	solitary	haemolymph	<i>Mamestra brassicae</i>	generalist	5

Table 1: Insect herbivores and their larval parasitoids used in this thesis

respectively (Table 1).

Direct defence: plant-mediated effects on herbivore performance

Brassicaceae specialists: interspecific plant variation

The performance of specialist herbivores on Brassicaceae was investigated on different cultivars (**chapters 3, 4 and 7**), on different populations of the same plant species (**chapters 4, 5 and 7**), and on different plant species (**chapters 3, 6 and 7**). *Plutella xylostella* as well as *P. brassicae* performed differentially on the various brassicaceous plant species. Females of *P. xylostella* attained higher biomass on the different mustard species, *Sinapis alba*, *Brassica nigra* and *B. juncea*, than on a Brussels sprout cultivar *B. oleracea*. *Pieris brassicae* developed faster and attained higher pupal mass on a wild *B. nigra* than on a wild *S. arvensis* population. The first question that can be raised is whether differences in GS composition and/or concentrations among the plant species are responsible for the differences in herbivore performance. GS analyses revealed considerable variation in GS composition and quantities among the various species (Table 2, see also Fig. 1). Different GS dominated the GS profile of each species: sinigrin in *B. nigra* (>95% of total GS content), sinalbin in *S. alba* (>75%) and gluconapin in *B. juncea* (>70%). The GS composition of *B. oleracea* is more complex and varies significantly among the different populations, but proportions of glucobrassicin in leaf tissues of herbivore-damaged plants tend to be high in all populations (50-90% of total GS content). Larvae of *P. xylostella* produce an enzyme in their gut that efficiently desulfates GS rendering them unsuitable substrates for myrosinase activity (Ratzka et al. 2002), thus preventing the formation of toxic hydrolysis products. The three different classes of glucosinolates, aliphatic, aromatic and indole GS, respectively, are all efficiently converted by this enzyme in *P. xylostella* (Ratzka et al. 2002). In *P. rapae*, the hydrolysis of GS into toxic (iso)thiocyanates is redirected and GS are converted into less toxic nitriles through the activity of a so-called nitro-specifier protein (NSP) (Wittstock et al. 2004; Agerbirk et al. 2006). A survey also demonstrated the presence of NSP in larvae of *P. brassicae* as well as in other species in the Pierinae feeding on GS-containing plants (Wittstock et al. 2004; Wheat et al. 2007). Since both herbivores have evolved efficient GS detoxification and excretion mechanisms, it is unlikely that differential performance of the two specialist herbivores can be attributed to differences in GS content and quantity.

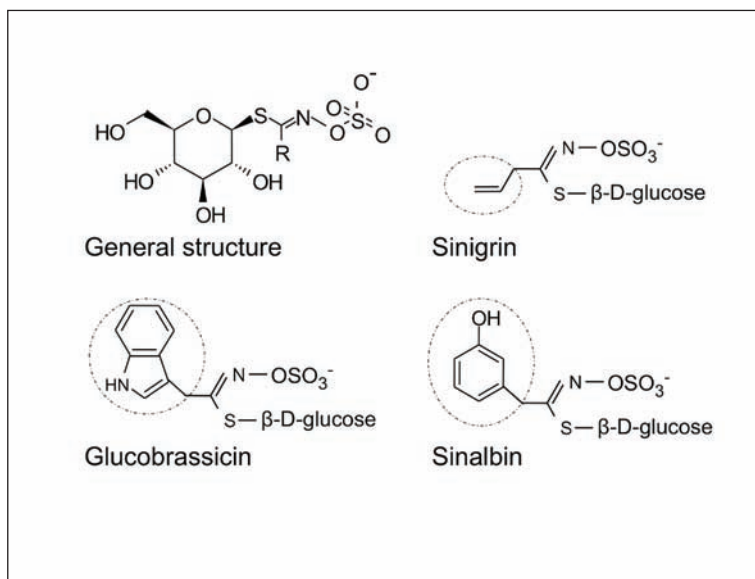
The development of specialist herbivores on brassicaceous plant species can also be negatively affected by high amounts of GS in their diet (Li et al. 2000; Agrawal and Kurashige 2003). However, total levels of GS in leaf tissues of the various

plant species used in my thesis were below the threshold levels necessary to reduce growth as was shown in the study by Li et al. (2000) and Agrawal and Kurashige (2003). In addition, Smallegange et al. (2007) reported that growth of *P. brassicae* is not negatively influenced by the relatively high levels of sinigrin present in *B. nigra* leaf tissues (Smallegange et al. 2007). Second and third instar larvae of *P. brassicae* move to the flowers, which contain significantly higher amounts of sinigrin than leaves. Moreover, the larvae attain higher biomass when they selectively feed on flowers than larvae feeding on leaf tissues (Smallegange et al. 2007). It is likely that the different plant species used in this thesis differed in other defence-related compounds such as chemicals that affect digestibility of the food. In addition, differences in primary plant metabolites, which are also very important for the development of the herbivore (Slansky 1993) may have further affected the development of the herbivores feeding on

Scientific name	Trivial name	Plant species	Range $\mu\text{mol/g}$ DW
Aliphatic GS			
3-Methylsulfinylpropyl GS	glucoiberin	<i>Brassica oleracea</i> <i>Brassica feral</i>	0.5 – 10 < 0.1
2-Propenyl GS	sinigrin	<i>B. nigra</i> <i>B. oleracea</i> <i>B. juncea</i> <i>Brassica feral</i>	10 – 70 1 – 10 1 – 10 < 0.1
4-Methylsulfinyl-3-butenyl GS	glucoraphanin	<i>B. oleracea</i> <i>Brassica feral</i>	0.5 – 5 < 0.1
3-Butenyl GS	gluconapin	<i>B. oleracea</i> <i>B. juncea</i> <i>Sinapis alba</i> <i>Brassica feral</i> <i>B. nigra</i>	0.1 – 40 15 – 30 0 – 2 0.1 – 1.0 < 0.5
2(R)-2-Hydroxy-3-butenyl GS	progoitrin	<i>B. oleracea</i> <i>Brassica feral</i> <i>S. alba</i>	0.5 – 5 0.1 – 5 0 – 5
2(S)-2-Hydroxy-3-butenyl GS	epiprogoitrin	<i>B. oleracea</i>	< 0.5
5-Methylsulfinyl pentyl GS	glucoallyssin	<i>Brassica feral</i>	0.1 – 1.0
4-Pentenyl GS	glucobrassicinapin	<i>Brassica feral</i> <i>B. juncea</i>	0.1 – 1.0 0.1 – 1.0
2-hydroxy-4-pentenyl GS	gluconapoleiferin	<i>Brassica feral</i>	0.1 – 1.0
Indole GS			
3-Indolylmethyl GS	glucobrassicin	<i>B. oleracea</i> <i>Brassica feral</i> <i>B. nigra</i> <i>B. juncea</i>	1 – 25 0.1 – 5 < 0.5 < 0.1
1-Methox-3-indolylmethyl	neoglucobrassicin	<i>B. oleracea</i> <i>B. juncea</i> <i>Brassica feral</i>	0 – 20 < 0.1 < 0.05
4-Hydroxy-3-indolylmethyl GS	4-hydroxy-glucobrassicin	<i>B. oleracea</i> <i>Brassica feral</i> <i>B. nigra</i>	0.1 – 1 < 0.05 < 0.1
4-Methoxy-3-indolylmethyl GS	4-methoxy-glucobrassicin	<i>B. oleracea</i> <i>B. nigra</i>	0.01 – 1 < 0.1
Aromatic GS			
2-Phenylethyl GS	gluconasturtiin	<i>B. nigra</i> <i>B. juncea</i> <i>Brassica feral</i>	< 0.5 < 0.5 < 0.1
Benzyl GS	glucotropaeolin	<i>S. alba</i>	1 – 25
4-Hydroxybenzyl GS	sinalbin	<i>S. alba</i> <i>S. arvensis</i>	10 – 80 1 – 25

Table 2: Glucosinolate (GS) concentrations in herbivore-damaged leaves of the different Brassicaceous plant species used in this thesis

Figure 1: General structure of glucosinolates and examples of the three different glucosinolates classes. Glucosinolates are divided into aliphatic (derived from methionine, e.g., sinigrin), indole (derived from tryptophan, e.g., glucobrassicin) and aromatic glucosinolates (derived from phenylalanine, e.g., sinalbin). The circles refer to the variable side chain denoted by 'R' in the general structure.



these plants.

Brassicaceae specialists: intraspecific plant variation

It is likely that plant chemistry differs less among populations of the same species than among populations of different species. For example, pupal mass and development time of *P. brassicae* was similar on three different *B. nigra* populations (**Chapter 7**). By contrast, *P. xylostella* females reared on the same populations developed significantly slower on one of the *B. nigra* populations. The performance of *P. xylostella* and *P. rapae* also differed when reared on a cultivar and a wild *B. oleracea* population (**Chapter 4**). *Pieris rapae* developed slower and attained lower adult mass on a wild *B. oleracea* population than on a cabbage cultivar, whereas these two plant populations only affected female adult biomass in *P. xylostella*. In **chapter 5**, I compared the performance of *P. rapae* on three different wild populations and a cultivar of *B. oleracea*. GS analyses in leaf tissues revealed considerable variation in GS concentrations among the different populations. Regression analysis of the different GS and the different performance parameters revealed a significant positive linear relationship between concentrations of the indole GS neoglucobrassicin and egg-to-pupa development time of *P. rapae*. The role of indole GS in defence against insect herbivory is poorly understood, in part, because levels of indole GS in foliage of cultivated *B. oleracea* and other Brassicaceous species are much lower than in wild *B. oleracea* plants (Giamoustaris and Mithen 1995; Rosa 1999; Traw 2002). Only the hydrolysis products of GS, such as isothiocyanates, have been shown to be active against specialist herbivores (Li et al. 2000; Agrawal and Kurashige 2003). Hydrolysis of aliphatic GS results in the

formation of stable isothiocyanates. Attempts to isolate stable isothiocyanate products of indole GS have not been successful (Bones and Rossiter 2006), suggesting that hydrolysis products of indole GS undergo further degradation. Whether indole GS and their breakdown products are detoxified by *Pieris* species in the same way as aliphatic and aromatic GS is unknown and merits further investigation.

These results show that the performance of even specialised insects can differ when reared on different crucifer species, but also when reared on different populations of the same species. Plant quality mainly affected development time and pupal/adult mass, whereas survival was high on all the different plant populations/species included in this thesis. Furthermore, the specialist herbivore species are differentially affected by their food plants, which suggests that the herbivores have variable nutritional requirements or else that they are differently affected by other defence-related compounds. Studies with artificial diets (e.g., Agrawal and Kurashige 2003) or the use of mutant plants that have been modified to enhance or eliminate a single trait (e.g., Burow et al. 2006) may help to better understand the relative effect of GS on insect performance.

Generalist herbivore: *Mamestra brassicae*

Generalist herbivores produce enzymes which can detoxify a wide range of substrates (Krieger et al. 1971), whereas specialists have evolved enzyme systems that can detoxify specific plant compounds that are associated with herbivore diet (e.g., Johnson 1999; Ratzka et al. 2002). Therefore, generalist herbivores are usually more sensitive to high levels of specific allelochemicals compared to specialist herbivores. (see e.g., Blau et al. 1978; Giamoustaris and Mithen 1995). In **chapters 4 and 5**, I compared the development of *M. brassicae* larvae on a cultivar and a wild population of *B. oleracea*. Adults of *M. brassicae* attained significantly lower body mass on the wild cabbage population “Old Harry” than on the cabbage cultivar (**Chapter 4**). Because larvae tend to move from plants of poor quality, larvae were maintained in Petri dishes in a second experiment in which I compared the performance of *M. brassicae* on different wild cabbage populations (**Chapter 5**). These precautions could not prevent dramatic effects on survival of this herbivore on one of the wild populations. None of the larvae survived on the “Winspit” population, whereas 60 % survived on the Old Harry population. Interestingly, *M. brassicae* survival was similar on the wild population “Kimmeridge” and on the cabbage cultivar. Regression analysis of the different GS in leaf tissues and survival of *M. brassicae* revealed a significant negative linear relationship between caterpillar survival and sinigrin, gluconapin and total GS, respectively. These results suggest that GS potentially play a role in reducing performance of *M. brassicae*.

In a recent study, van Leur et al. (2008) examined the effects of two *Barbarea vulgaris* ecotypes on the performance of *M. brassicae* larvae. The two ecotypes are dominated by different GS, glucobarbarin in the so-called 'BAR'-chemotype and gluconasturtiin in the so-called 'NAR'-chemotype. Survival of *M. brassicae* was demonstrated to be low on the BAR-chemotype, whereas the larvae on the NAR-chemotype all survived and grew exponentially during the 8-day feeding bioassay (van Leur et al. 2008). In addition, survival of *M. brassicae* is low (<10% larvae survive to pupation) when the larvae feed on *B. nigra* or *S. arvensis*, in which sinigrin and sinalbin, respectively, are the dominant GS (J. Harvey, unpublished data). Thus, although not all GS are equally toxic to *M. brassicae*, development of this generalist herbivore can be strongly reduced by GS in their diet. Moreover, in *M. brassicae* food plant quality not only affected body mass and development time, as was demonstrated for the specialist herbivores, but also had a profound impact on a more direct indicator of fitness, i.e., survival.

In summary, I have shown that interspecific and intraspecific variation in plant quality can differentially affect the performance of generalist and specialist herbivores. Moreover, plant quality had a more pronounced influence on the performance of the generalist *M. brassicae* than on the specialist herbivores, *P. rapae*, *P. brassicae*, and *P. xylostella*. In the specialists, differences in plant quality mainly affected development time and pupal/adult size, whereas in *M. brassicae* survival was also strongly influenced by plant quality. Difference in herbivore performance on domesticated and wild conspecific plants depended on the plant species. Differences in performance were only marginal when the herbivores were reared on cultivated or wild populations of *B. nigra*. On *B. oleracea* on the other hand, variation in herbivore development was more pronounced, both between cultivated and wild populations as well as among the wild populations. Directional selection on leaf structures in *B. oleracea* has resulted in considerable changes in GS chemistry (Kushad et al. 1999; Rosa 1999). GS contents in *B. nigra* cultivars are less well studied, but GS concentrations in *B. nigra* seeds tend to be relatively high compared to GS levels in seeds of *B. oleracea* cultivars (Velasco and Becker 2000). Interestingly, GS chemistry in *B. oleracea* populations varied over relatively small spatial scales (5-15 km), whereas the two wild *B. nigra* populations, which were collected in the Netherlands and Italy, respectively, were similar in GS content. Thus, the effects of domestication on plant GS chemistry and concomitant effects of these changes on herbivore performance depend on the plant species as well as the direction of breeding programs.

Direct defence: plant-mediated effects on the performance of larval endoparasitoids

Specialist parasitoids of herbivores specialised on brassicaceous plants

Insect herbivores obtain their nutrition directly from their food plants. Consequently, parasitoids of these herbivores obtain their nutrition indirectly from the plants (Bottrell et al. 1998; Turlings and Benrey 1998). It has been shown that plant allelochemicals may not only negatively affect the development of herbivores but also that of their parasitoids (reviewed by Hunter 2003; Ode 2006). I investigated the performance of different larval endoparasitoids developing in hosts that are specialists on brassicaceous plant species. As for the herbivores, I studied the effect of intraspecific and interspecific variation in plant quality on parasitoid performance. First (**chapter 3**), I investigated the development of *Diadegma semiclausum*, which is a specialist solitary endoparasitoid of *P. xylostella* on two different cultivated brassicaceous plant species, *B. oleracea* (Brussels sprouts) and *S. alba* (white mustard). In this study we found that, although performance of the host was different on the two plant species, performance of the parasitoid was similar, irrespective of the food plant species on which the host had been feeding. In **chapter 7**, I compared the performance of *D. semiclausum* and *Cotesia glomerata* from their respective hosts on different *B. nigra* populations and a *B. juncea* cultivar. Although plant population had a significant effect on development time and adult mass of both parasitoid species, multiple comparisons among the means did not reveal consistent differences between populations. These results suggest that the effects of food plant quality on performance of the parasitoids are not very strong. Both parasitoid species are specialists with respect to their host range and their hosts are specialists with respect to their food-plant range. *Diadegma semiclausum* is considered to be a specialist larval endoparasitoid of *P. xylostella* (Talekar and Shelton 1993) and *C. glomerata* is a specialist on a few species in the Pierinae (Brodeur and Vet 1995). Efficient detoxification and excretion of GS by the hosts minimises exposure of the parasitoid larvae to these plant-derived compounds.

Interestingly, intraspecific variation in food plant quality among *B. oleracea* populations affected development of the larval endoparasitoids. In **chapter 4**, the performance of *D. semiclausum* was compared when its host had been feeding on a cultivar and a wild cabbage population. Adult *D. semiclausum* were significantly smaller on the wild cabbage population than on the cabbage cultivar. Performance of *Cotesia rubecula* also varied when reared from its host, *P. rapae*, on different wild and

cultivated cabbage populations (**chapter 5**). In addition, adult body mass of *C. glomerata* was lower on a Sicilian *B. nigra* population than on a Dutch population and a cultivar of this plant species (**chapter 7**). However, when we compared different populations of *B. nigra*, both cultivated and wild (**chapter 7**), development time and adult biomass of *D. semiclausum* was similar on the three *B. nigra* populations. Thus, variation in plant quality among populations may influence parasitoid performance depending on the plant species, but this also depends on the host-parasitoid system. In all of the parasitoid species mentioned above plant quality only affected indirect fitness correlates (biomass and development time), whereas survival was high on all the plant species/populations.

As was discussed with the herbivores, the question can be raised whether GS in the host's diet affect parasitoid performance. All three herbivorous hosts (*P. xylostella*, *P. rapae* and *P. brassicae*) efficiently detoxify and excrete GS (Ratzka et al. 2002; Wittstock et al. 2004; Agerbirk et al. 2006; Wheat et al. 2007). Therefore, exposure to GS or their breakdown products by parasitoid larvae that develop inside these hosts is expected to be low. In addition, parasitoid species that attack hosts with a restricted food range may have evolved their own detoxification mechanisms. *Diadegma semiclausum* is an obligate tissue feeder and has to consume the host including the gut and its contents before it can pupate. Early larval stages of this parasitoid species primarily feed on haemolymph. However, during the destructive feeding phase, the larvae may be exposed to toxic allelochemicals that are still present in the gut. Little is known about the detoxification of plant derived allelochemicals by larval endoparasitoids (Ode 2006). Exposure to plant derived compounds is expected to be even less in parasitoids such as *Cotesia* species that mainly feed on haemolymph and fat body (Harvey and Strand 2002) and which may avoid feeding on host tissues in which plant-derived allelochemicals are stored.

Generalist parasitoid on a herbivore that is specialised on brassicaceous plants

In **chapter 4**, I compared the development of two closely related parasitoid species, *D. semiclausum* and *D. fenestrale* reared on the same host, *P. xylostella*, on wild and cultivated cabbage. These two parasitoid species differ in host specialisation, with *D. semiclausum* being restricted to *P. xylostella* and *D. fenestrale* attacking several other hosts feeding on non-brassicaceous plant species (Azidah et al. 2000; Legaspi 1986). Although both parasitoid species developed more poorly in hosts on the wild cabbage population, *D. fenestrale* was more strongly affected by plant population than *D. semiclausum*. Moreover, survival was extremely low (5% of the larvae survived to adulthood) when *D. fenestrale* was reared from *P. xylostella* on *B. nigra*,

which is a high quality food plant for healthy *P. xylostella* larvae. In hosts parasitised by *D. fenestrale*, mortality mainly occurred just before the parasitoids would have been expected to pupate. When the larvae of *D. fenestrale* are feeding on haemolymph, they are not exposed to GS, because *P. xylostella* caterpillars detoxify GS in their gut. However, during the destructive feeding phase, the parasitoid larvae consume the host including the gut and its contents, and this is when the toxic effects of plant allelochemicals on non-adapted parasitoids could occur. Total GS concentrations are relatively high in *B. nigra* plants compared to total GS concentration in the feral *Brassica* and the two *B. oleracea* populations. Differences in total GS could therefore explain the higher mortality of *D. fenestrale* when the host feeds on *B. nigra* compared to the three other plant populations used in this study. In contrast, *D. semiclausum* is adapted to or can tolerate plant-derived compounds in the host's gut.

Specialist parasitoid of a generalist herbivore

Thus far I compared the performance of parasitoids on hosts that are specialists on brassicaceous plants. In **chapter 5**, I also investigated the development of a parasitoid species (*Microplitis mediator*) attacking larval stages of a polyphagous host (*M. brassicae*) on three wild and a cultivated population of *B. oleracea*. Survival of *M. mediator* was greatly affected by plant population and reflected that of its host. Because *M. brassicae* has not evolved an efficient detoxification mechanism to deal with the adverse effects of GS, larvae of *M. mediator* may be exposed to GS or their breakdown products that have passively diffused into the host haemolymph. Alternatively, parasitoid performance is indirectly affected by the food plant of the host through reduced growth of the herbivore itself.

In conclusion, it has been demonstrated that the performance of the host and its parasitoid are often positively correlated in specialised herbivore-parasitoid associations, although the adverse effects of food plant characteristics on performance are often less pronounced in the parasitoid than in the herbivore. Furthermore, in specialised herbivore-parasitoid associations, plant quality mainly affects indirect indicators of fitness such as body mass and development time in both the herbivores and their parasitoids. In generalist parasitoids that attack hosts with a restricted food range, such as the *P. xylostella*-*D. fenestrale* system, the effect of plant quality on parasitoid performance does not necessarily reflect that of the host. In herbivore-parasitoid systems where the host is polyphagous and is not adapted to deal with plant allelochemicals such as GS, performance of the host and the parasitoid are usually correlated. In these associations, plant quality may not only affect body mass and development but may also have a pronounced effect on survival.

Indirect defence: parasitoid attraction to different mustard populations

Is there a conflict between indirect and direct defence?

Studies on plant defences are either focused on direct defence or indirect defences. However, the same genes may be involved in regulating both direct and indirect defence traits. In other words, a plant with high levels of direct defences may also have high levels of indirect defences and *vice versa* (e.g., Thaler et al. 2002). However, cross talk between signalling pathways or differential expression of genes may prevent opposing effects in the expression of different defence traits (Kahl et al. 2000; De Vos et al. 2005). Several studies have shown that allelochemicals in the host diet can also affect the performance of larval endoparasitoids (Campbell and Duffey 1979; Barbosa et al. 1986; Ode et al. 2004). A conflict may arise when a herbivore's enemy is attracted to plants that have a negative impact on its own development or that of its progeny (Thaler 1999; Havill and Raffa 2000). In part II of my thesis I investigated whether direct and indirect plant defences are compatible. In other words, is there a correlation between preference and performance in parasitoids?

In **chapter 6**, I compared the attraction of female *C. glomerata* to two different brassicaceous plant species, *B. nigra* and *S. arvensis* when damaged by *P. brassicae* caterpillars. Female wasps preferred to land on host-infested *B. nigra* plants. Although performance (body mass and development time) of the parasitoid was similar on the two plant species, unparasitised *P. brassicae* larvae developed faster and attained higher pupal mass on *B. nigra* plants. This study revealed no conflicts between direct and indirect defences; the parasitoid female preferred the food plant on which the host performed best.

The evidence that plants release volatile chemicals in response to herbivore damage that attract parasitoids and predators of herbivores is overwhelming (reviewed by Dicke 1999a; Heil 2008; Takabayashi et al. 2006; Turlings and Benrey 1998). However, whether the production of HIPV's is an evolutionarily reinforced trait remains the subject of debate (van der Meijden and Klinkhamer 2000; Hoballah and Turlings 2001; Janssen et al. 2002; Heil 2008). If it is, we would expect genetic variation in the production of volatile blends among individual plants and between different plant populations, as has been demonstrated with direct defence (Brower et al. 1972; Vrieling et al. 1991; Moyes et al. 2000; Zangerl and Berenbaum 2005). Plant populations growing in habitats where the intensity of herbivore attack or in which the reliability of "alarm signals" to natural

enemies varies, may differ in their defence genotype (van der Meijden and Klinkhamer, 2000). The aim of the study presented in **chapter 7** was to investigate whether different populations varied in their ability to attract parasitoids and whether attraction was positively correlated with offspring performance.

In **chapter 7**, I conducted similar experiments as in chapter 6. I compared the attraction of *C. glomerata* and *D. semiclausum* to two wild *B. nigra* populations that originated from different geographical regions (Sicily and the Netherlands) as well as a cultivar which has been subjected to artificial selection. As an out-group, I included a hybrid of *B. nigra* and *B. rapa* (*B. juncea*). The two parasitoid species were differentially attracted to the four plant populations. Female *D. semiclausum* preferred host-infested *B. juncea* plants over host-infested *B. nigra* plants and did not discriminate among the three *B. nigra* populations. In contrast, female *C. glomerata* were less attracted to the Sicilian *B. nigra* population. Headspace analysis of *P. xylostella*-infested plants mainly revealed quantitative differences among plants of different *B. nigra* populations, whereas *B. juncea* plants also emitted volatile compounds that were not detected in the odour blend of *B. nigra* plants. Apparently, the volatiles that are important for the attraction of *D. semiclausum* did not differ among the *B. nigra* populations. We did not chemically analyse the volatile blend emitted by plants that were damaged by *P. brassicae*. However, earlier studies have revealed that the volatile blends emitted by the same *B. oleracea* cultivar infested by different herbivores mainly differed in volatile quantity and not quality (Blaakmeer et al. 1994; Geervliet et al. 1997).

More importantly, *C. glomerata* varied in its response to the different mustard populations when compared with *D. semiclausum*. These results indicate that the two parasitoid species may use different compounds or mixtures of compounds as foraging cues. However, the results in this study may be confounded, because *C. glomerata* females had an oviposition experience on a host feeding on Brussels sprouts prior to release in the behavioural bioassay. The response of female parasitoids to HIPV's can be modified through associative learning (Vet and Dicke 1992; Vet et al. 1995). I cannot exclude the possibility that this oviposition experience has resulted in a response that is different from naïve (=without oviposition experience) females (Geervliet et al. 1998). If parasitoids exert selection pressure on plants to produce more attractive HIPV's, then naïve wasps should be able to discriminate between individuals that differ in their volatile emissions. If learning plays an important role in foraging behaviour of parasitoids then selection on the production of a more attractive blend is expected to be low. The parasitoid female first has to find this plant before it can associate the emitted volatile blend with a host and attraction to this specific odour is then reinforced. Little is known about the importance

of learning on foraging behaviour of parasitoids in agricultural systems (Geervliet et al. 2000) and even less is known about foraging behaviour of parasitoids in natural plant communities.

The three different plant species *B. nigra*, *B. juncea* and *S. arvensis* appeared to be very suitable plants for development of both herbivores, *P. xylostella* and *P. brassicae* as well as their parasitoids, *D. semiclausum* and *C. glomerata*, respectively. As a result, direct and indirect plant defence traits in the three mustard species did not have opposing effects on the two parasitoid species. It would be interesting to compare the performance and attraction of a parasitoid on plant species or plant populations on which development is negatively affected by plant quality as was found for *C. rubecula* on two wild *B. oleracea* populations (**chapter 5**). The attraction of *C. rubecula* to HIPV's emitted by the three wild *B. oleracea* populations is currently being studied. These studies should also include the attraction of parasitoids like *M. mediator* that parasitise polyphagous hosts and are more strongly affected by food plant quality. However, learning is expected to play a more important role in the foraging behaviour of generalist than of specialist parasitoids (Vet and Dicke 1992; Steidle and van Loon 2003). Naïve generalist parasitoids may respond to general volatile plant signals and may only learn to discriminate among more subtle differences in volatile blends after oviposition experience.

Cultivation

Cultivation has generally been aimed at enhancing a trait or a suite of traits in the plant, such as the production of specific plant organs or structures. Importantly, this may or may not be compatible with direct or indirect defences. For instance, one of the most important traits in vegetable crops is their taste. Selection for this trait may be in direct conflict with direct defence depending on whether this trait is emphasised (e.g., mustard condiments) or not (e.g., cabbage). As a consequence of artificial selection, levels of primary and secondary plant compounds in crop plants may have changed in such a way that these plants have become more susceptible to insect attack. For example, levels of GS in *B. oleracea* cultivars are often reduced compared with wild conspecific plants (Rosa 1999; Moyes et al. 2000; Kushad et al. 2004). I compared performance of herbivores and parasitoids on cultivated and wild populations of *B. oleracea* (**chapter 4** and **5**) and *B. nigra* (**chapter 7**). Differences among cultivated and wild *B. oleracea* had a more pronounced effect on the performance of the herbivores and their parasitoids than differences among cultivated and wild populations of *B. nigra*. Moreover, performance of the generalist *M. brassicae* was more

strongly affected by the different wild *B. oleracea* populations than the performance of the specialists, *P. rapae* and *P. xylostella*.

Clearly, artificial selection in *B. oleracea* has resulted in a reduction in GS levels in leaf tissues, whereas in *B. nigra* concentrations of foliar GS are relatively high and are similar to levels found in wild populations of this plant species. Artificial selection on specific plant traits in both plant species differs considerably. In *B. oleracea* selection is often aimed at enlarging foliar structures, whereas in *B. nigra* selection is aimed at seed production. These differences in breeding programs may have had variable effects on chemical properties of the plants, which in turn may affect insect communities associated with these crop plant species. In addition, artificial selection in vegetable crops may have resulted in reduced levels of allelochemicals, either intentionally or accidentally. The quality and quantity of HIPV's may have remained more similar to HIPV's emitted by wild conspecifics, although some studies showed that different cultivars vary in the production of HIPV (Loughrin et al. 1995; Geervliet et al. 1997; Gouinguene et al. 2001). Thus, potential conflicts between direct and indirect defences may have been reduced in cultivated plants. It remains to be determined how this conflict is resolved in unmanaged natural ecosystems. For example Gauld et al. (1992) revealed that endoparasitoid lineages in tropical regions are species-poor compared with temperate regions, a pattern that is opposite to most global patterns for biodiversity (Gaston 2007). Gauld et al. (1992) argued that tropical plants are extremely toxic, limiting adaptive radiation amongst endoparasitoids. Conflicts between direct and indirect defences may rarely occur in agricultural systems where direct defences have been reduced.

Foraging behaviour of parasitoids: beyond the Y-tube olfactometer and windtunnels

Studies on the attraction of parasitoids to HIPV's in Y-tubes and windtunnels certainly have their merits. It is possible to separate the response to volatiles from responses to e.g., visual cues, and volatiles can also be offered in a controlled manner. In addition, in these highly sophisticated instruments it is possible to test the attraction of insects to pure compounds. The question is to what extent results obtained from Y-tubes and windtunnels can be translated to natural environments. A few studies have shown that herbivore-infested plants selectively attract parasitoids in the field (De Moraes et al. 1998; Geervliet et al. 2000). In addition, the manipulation of volatile emissions using chemical elicitors, intercropping, mutant plants or synthetic volatiles compounds resulted in increased rates of parasitism or predation in field studies (Khan et al. 1997; Thaler 1999; Halitschke et al. 2008).

Most of these studies were performed using monocultures often with crop plant species. However in nature, parasitoids have to search for hosts in diverse environments consisting of many different plant species, which may be damaged by several attackers. Many studies in agro-ecological research assume that tritrophic interactions (e.g., plant-herbivore-parasitoid interactions) function similarly in managed and unmanaged ecosystems (Barbosa 1993). However, the validity of these assumptions has rarely been empirically tested. The ‘resource concentration’ hypothesis, which was introduced by Root (1973), stated that specialised herbivores attain higher densities in monocultures, because they are more likely to find and remain on host plants that are growing in pure stands. Likewise, given that they evolved in more complex natural ecosystems, parasitoids are often less challenged in finding hosts in agricultural monocultures and frequently overexploit host populations in cropping systems. In **chapter 8**, I compared the foraging behaviour of *D. semiclausum* in a slightly more natural setting. The set-up consisted of cabbage plants that were either interspersed with a different brassicaceous plant species (*S. alba*) or the cereal species, *Hordeum vulgare* (barley). Structural characteristics of the habitat were also varied. The aim of the study was to determine whether composition and spatial arrangements of plants in a habitat affected the tendency of a parasitoid to find a host-infested plant. The efficiency of the parasitoid to find the host-infested *B. oleracea* plant was reduced in a set-up that consisted of both *B. oleracea* and *S. alba*. Bukovinszky et al. (2005) showed that undamaged *S. alba* and host-damaged *B. oleracea* plants are equally attractive to *D. semiclausum* females in a Y-tube olfactometer. If parasitoids are attracted to some plant types than others in nature, then hosts on less attractive types are more likely to escape parasitism. In natural plant communities, parasitoid foraging efficiency may be further reduced when plants are damaged by both hosts and non-hosts, although it has been demonstrated that specialist parasitoids and predators can often discriminate between volatile blends emitted by plants damaged by hosts and non-hosts (Shiojiri et al. 2001; de Boer et al. 2004;). Increasing habitat complexity may reduce the efficiency of a foraging parasitoid. Reduced complexity in agricultural fields may explain why parasitoids easily exploit hosts in these systems (Bukovinszky 2004). Comparison of the foraging behaviour and population dynamics of hosts and their parasitoids in managed agricultural and unmanaged natural ecosystems may provide new insights into the mechanisms underlying tritrophic interactions (e.g., Ohsaki and Sato 1999).

Future directions

Those who study mechanisms involved in plant-insect interactions should place their results in a broader ecological and/or evolutionary frame work. One of the most enduring debates in ecology has concerned the relative importance of 'top-down' (natural-enemy controlled) versus 'bottom-up' (plant controlled) forces in regulating the structure and function of ecological communities. Hairston et al. (1960), Price et al. (1980) and Hunter and Price (1992) argued that a more complete understanding of assembly rules in ecological communities needed to incorporate the role played by natural enemies in reducing damaging herbivore populations. However, this view has been widely challenged (Hartley and Jones 1986; Strong 1992; Polis and Strong 1996; Schmitz et al. 2000; Shurin et al. 2002). Clearly, the importance of top-down control has been proven in agricultural systems, and underpins biological control programs. In this context, indirect plant defences are vitally important (Khan et al. 1997; Geervliet et al. 2000). However, much evidence suggests that bottom-up forces play a much more important role in regulating herbivore populations in more structurally (and chemically) complex natural systems (Strong 1992; Polis and Strong 1996). Even recent studies broadly arguing in support of trophic cascades in terrestrial systems (e.g., Schmitz et al., 2000) acknowledge that top-down regulation may be more important in simple ecological communities dominated by one or only a few plant species (such as in agricultural landscapes). Further, they suggest that most examples of trophic cascades in natural ecosystems involve vertebrate predator-prey systems. One of the most important factors to remember is that plant food is at best suboptimal, and at worst, toxic for many herbivores. Comparison of the foraging behaviour and population dynamics of hosts and their parasitoids in managed agricultural and unmanaged natural ecosystems may provide new insights into the mechanisms underlying tritrophic interactions (e.g., Ohsaki and Sato 1999) and may help to reveal the importance of bottom-up and top-down control of arthropod herbivores.

Acknowledgements

I thank Marcel Dicke and Jeff Harvey for constructive comments on draft versions of the manuscript.

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SUMMARY

Plants have evolved a range of defence traits that prevent or reduce the risk of attack by insect herbivores. Usually, these preventive measurements are classified as direct and indirect plant defences. Direct defence traits hamper or reduce performance and behaviour of the herbivores. For example, morphological plant characteristics may impede herbivore movement on the plant, whereas plant chemicals may compromise the growth and development of the herbivore. Indirect defence promotes the efficiency of natural enemies such as predators and parasitoids to control the herbivores. Special plant structures and alternative food sources encourage natural enemies to live on the plants where it has been suggested that they function as 'bodyguards'. Furthermore, most plants release volatile chemicals in response to herbivore damage, which are attractive to parasitoids and predators of herbivores. In this thesis, I focused on chemical plant defences, i.e. the production of allelochemicals (direct defence) and volatile organic compounds that are produced in response to herbivore damage (indirect defence).

I have chosen to work with plant species in the Brassicaceae for three main reasons. First this family includes species with a wide array of life-history strategies; second, insects associated with brassicaceous plants are well-known; and finally, because it has given rise to important crops. These crop varieties include cabbage vegetables (*Brassica oleracea*), oilseed varieties (e.g., *Brassica juncea* and *Brassica napus*) and mustard condiments (*Brassica nigra* and *Sinapis alba*). In addition, several wild brassicaceous plant species grow naturally in the Netherlands. However, through artificial selection, the chemistry in crop plants may have been modified. All brassicaceous plant species produce glucosinolates (GS), secondary metabolites that play an important role in defence against insect herbivores. Herbivorous pests and their natural enemies are well studied in Brassicaceous crop plant species. To better understand the evolution of plant defences against insect herbivores, it is important to study plant defences also in wild plant species in which defence traits are intact.

Insect herbivores such as *Pieris rapae* and *Plutella xylostella* mainly feed on brassicaceous plant species. Specialist herbivores like *P. rapae* and *P. xylostella* have evolved special enzymatic adaptations to excrete and/or detoxify specific plant compounds that are associated with the herbivore's diet such as GS in the Brassicaceae. Generalist herbivores such as *Mamestra brassicae* feed on many plant species in several plant families and produce enzymes that can detoxify a wide range of substrates. Generalist herbivores are usually more sensitive to high levels of specific allelochemicals compared to specialist herbivores. Plant allelochemicals such as GS may not only affect growth and survival of the herbivores, but they may also reduce the performance of the herbivore's natural enemies such as parasitoids. Like the herbivores differ in food plant specialisation, parasitoid species differ in host specialisation. I have compared the performance of generalist and specialist herbivore and parasitoid species on several cultivated and wild conspecific plant populations. I expected that the specialist herbivores and parasitoids would be less affected by differences in host plant chemistry among the various Brassicaceous plant species than generalists.

Effect of food plant on the performance of herbivores and their parasitoids

In chapters 3-7, I investigated the performance of different herbivores and their endoparasitoids on wild and cultivated conspecifics of cabbage (*B. oleracea*) and several mustard species (*B. nigra*, *B. juncea*, *Sinapis arvensis*, and *S. alba*). Specialist herbivore-parasitoid systems did not appear to be strongly affected by variation in quality of the food plant. All of the mustard species appeared to be very suitable food plants for both the herbivores (*P. xylostella* and

Pieris brassicae) and their parasitoids (*Diadegma semiclausum* and *Cotesia glomerata*, respectively). The specialists were not affected by the relatively high GS concentrations in leaf tissues of the mustard species both in the wild and cultivated populations. The effect of food plant quality on performance of the parasitoids was less pronounced than was found for the herbivores. Secretion and/or detoxification of plant allelochemicals by the host may dilute the effect of these compounds on the development of the parasitoid. Alternatively, specialised parasitoids, like their hosts, have evolved various mechanisms to deal with toxic secondary plant compounds.

Populations of wild cabbage, *B. oleracea*, grow naturally along the Atlantic coastlines of the United Kingdom and France. Previous studies have reported differences in the concentrations of GS among populations of *B. oleracea* over a limited spatial gradient of < 50 km. In chapters 4 and 5, I used three of the Dorset (England) populations and a Brussels sprout cultivar in developmental studies with herbivores and their parasitoids in a greenhouse. The specialist herbivores *P. rapae* and *P. xylostella* performed better on the cultivar than on some of the wild cabbage populations. Development of the parasitoids *C. rubecula* and *D. semiclausum* reflected that of their hosts *P. rapae* and *P. xylostella*, respectively. As was found in the studies with different mustard species, plant quality only affected size and development time.

I also compared the development of a generalist herbivore *M. brassicae* and its fairly specialised parasitoid *Microplitis mediator* on different wild cabbage populations and a cultivar. Development of *M. brassicae* and *M. mediator* was more strongly affected by differences in quality among the wild *B. oleracea* populations than that of the specialist *P. rapae* when reared on the same populations. Larval development of *M. brassicae* was faster and pupae were heavier on the cultivar and the wild “Kimmeridge” population than on the wild “Old Harry” population. None of the larvae survived on the third wild population “Winspit”. Survival of *M. mediator* reflected that of its host.

Chemical analyses of GS in leaf tissues revealed considerable variation in concentrations of the different GS compounds. Furthermore, feeding damage by *P. rapae* induced the production of indole GS more strongly in the wild populations than in the cultivar. Total levels of GS were 2-4 times higher in *P. rapae*-damaged wild cabbage populations than in the Brussels sprout cultivar. I found strong negative correlations between the survival of *M. brassicae* and the two GS compounds sinigrin and gluconapin as well as total levels of GS, respectively. For *P. rapae*, I only found a positive correlation between egg-to-pupal development time and concentrations of the indole GS neoglucobrassicin.

In chapter 4, I also investigated the development of the

generalist parasitoid *Diadegma fenestrale* in *P. xylostella* larvae that were either feeding on cultivated or wild *B. oleracea* or *B. nigra* (black mustard). *Diadegma fenestrale* is closely related to *D. semiclausum* but has a wider host range than the latter species. In contrast to the development of *D. semiclausum*, the development of *D. fenestrale* was strongly affected by the food plant of the host. Survival was higher on the cultivar than on the “Old Harry” population. However, only 5% of the parasitised hosts developed into adult parasitoids on *B. nigra*, the food plant on which the unparasitised hosts were the heaviest.

Thus, specialist herbivore-parasitoid systems on Brassicaceous plants can be affected by intra- and interspecific variation in food-plant quality. Plant-mediated effects on performance in these systems primarily concern development time and pupal/adult weight. Differences in plant quality had a more pronounced effect on the generalist herbivore, *M. brassicae* and its parasitoid *M. mediator*. In this system, variation in plant quality not only affected development time and body mass, but also strongly influenced survival of the insects. Furthermore, performance of *M. brassicae* correlated with total levels of GS in the food plant, whereas for the specialists this correlation was absent or not very strong.

Conflicts between direct and indirect defences?

Direct and indirect defences may not act independently; a plant with high levels of direct defences may also have high levels of indirect defences and *vice versa*. A conflict between direct and indirect defences may arise when a parasitoid female is attracted to a plant on which the development of her offspring is compromised. As I showed, plant quality may not only affect the performance of herbivores, but also that of their endoparasitoids. In chapters 6 and 7, I investigated whether direct and indirect plant defences are compatible, i.e. are parasitoid females attracted to plants that provide the best hosts for the development of their progeny. I studied the performance of two different specialist herbivore-parasitoid systems (*P. xylostella*-*D. semiclausum* and *P. brassicae*-*C. glomerata*) on three different mustard species (*B. nigra*, *B. juncea* and *S. arvensis*). In addition, I recorded the attraction of the two parasitoid species to volatiles from host-infested plants. No conflicts between direct and indirect defences were revealed. Parasitoid females were attracted to plants on which their offspring was not negatively affected by the food plant quality of the host.

Defences in cultivated versus wild conspecifics

Cultivation has generally been aimed at enhancing a trait or a suite of traits in the plant, such as the production of specific plant organs

or structures. Importantly, this may or may not be compatible with maintaining the plant's direct and/or indirect defences. As a consequence of artificial selection, levels of primary and secondary metabolites in crop plants may have changed in such a way that these plants have become more susceptible to insect attack. I showed that GS concentrations are considerably lower in a Brussels sprout cultivar than in wild cabbage populations. Moreover, herbivores performed better on the cultivar than on some of the wild *B. oleracea* populations. The performance of the herbivores on the various mustard species was only marginally affected by differences in food-plant quality, despite the relatively high GS concentrations in mustard leaf tissues. In cabbage cultivars, selection on leaf structures has resulted in reduced foliar levels of GS compared to wild conspecifics, whereas selection on seeds in mustard and oilseed varieties has only marginally altered GS concentrations in leaf tissues. Whether artificial selection has resulted in chemical changes with concomitant effects on the performance of their associated insects depends on the plant trait that has been selected for. It is not clear to what extent plant breeding programs have altered the production of herbivore induced plant volatiles (HIPV's). Conflicts between direct and indirect plant defences are less likely to occur in agroecosystems, because direct chemical defence levels are often reduced in cultivated plant species.

Foraging behaviour of parasitoids: beyond the Y-tube olfactometer and windtunnels

Many studies comparing parasitoid attraction to HIPV's are conducted in Y-tube or windtunnel experiments. However, under natural conditions parasitoids have to search for hosts in more complex heterogeneously structured environments with different plant species that can be attacked by several herbivores. In the last chapter of my thesis, I studied parasitoid foraging behaviour in a slightly more complex setting. The basic set-up consisted of only *B. oleracea* plants (Brussels sprouts) with only the central plant containing hosts. Female *D. semiclausum* wasps were observed continuously until they had located the target plant and had parasitised five out of the 15 *P. xylostella* larvae. Foraging behaviour was also studied in set-ups in which the complexity was increased by adding either a second brassicaceous plant species, *S. alba* (white mustard) or a non-brassicaceous species, *Hordeum vulgare* (barley). Female *D. semiclausum* took significantly more time to find the host-infested *B. oleracea* plant in the set-up consisting of *B. oleracea* plants interspersed with either *S. alba* or *H. vulgare* than in the monospecific set-up with only *B. oleracea* plants. Thus, *B. oleracea* plants may provide the host *P. xylostella* with a chemical refuge against parasitism in the presence of highly attractive *S. alba* plants. Barley plants may

impose a physical barrier that hampers parasitoid movement. These results suggest that parasitoid responses measured in windtunnels and Y-tube olfactometers do not directly translate into foraging decisions made by females in natural communities.

Comparison of the foraging behaviour and population dynamics of hosts and their parasitoids in managed agricultural and unmanaged natural ecosystems is likely to provide new insights into the mechanisms underlying tritrophic interactions and will help to reveal the importance of bottom-up and top-down control of arthropod herbivores.

SAMEN- VATTING

Tritrofe interacties in wilde en gecultiveerde kruisbloemige planten

Planten worden belaagd door een divers scala aan organismen, zoals insecten en ziekteverwekkers. Planten staan echter niet volkomen weerloos tegenover deze potentiële bedreigingen. Zo kunnen haren, stekels en andere morfologische aanpassingen de verplaatsing van herbivore (=planten etende) insecten op de plant bemoeilijken. Daarnaast kunnen chemische stoffen in de plant de groei van herbivore insecten vertragen en hun overleving verminderen. Bovengenoemde voorbeelden zijn vormen van directe plantenverdediging, d.w.z. planteneigenschappen die de herbivoren negatief beïnvloeden. Naast een direct verdedigingsarsenaal, kunnen planten ook de 'hulp inroepen' van de natuurlijke vijanden van de herbivoren, zoals predatoren en sluipwespen. Zo kunnen planten alternatief voedsel en een schuilplaats aanbieden aan deze 'bodyguards', zodat deze in de buurt van de plant blijven. Daarnaast verspreiden planten die zijn beschadigd door herbivoren vluchtige stoffen die aantrekkelijk zijn voor sommige sluipwespen en predatoren. Het bevorderen van de effectiviteit van natuurlijke vijanden wordt indirecte plantenverdediging genoemd. In dit proefschrift heb ik mij gericht op chemische plantenverdediging, zowel direct als indirect.

Ik heb gekozen om te werken aan planten in de familie van de kruisbloemigen (Brassicaceae) omdat deze familie economisch belangrijke cultuurgewassen heeft voortgebracht en ook vele soorten bevat die in Nederland in het wild voorkomen. Zo heeft plantenveredeling geleid tot een grote verscheidenheid aan koolvariëteiten (bloemkool, spruitkool, broccoli, witte en rode kool e.d.) die allemaal afgeleid zijn van dezelfde plantensoort (*Brassica oleracea*). Daarnaast bevat deze familie soorten die geteeld worden vanwege hun oliehoudende zaden (*Brassica juncea* en *Brassica napus*) en worden de zaden van andere soorten gebruikt voor de productie van mosterd (*Sinapis alba*, *Brassica nigra*). Veredeling gericht op het vergroten van bepaalde plantenstructuren, zoals in *B. oleracea*, heeft echter niet alleen geleid tot een grote diversiteit aan cultivars, maar ook tot een reductie van glucosinolaat (GS) concentraties. GS zijn secundaire plantenstoffen karakteristiek voor de Brassicaceae. Secundaire plantenstoffen zijn stoffen die geen directe rol spelen in groei, onderhoud en reproductie van de plant. Deze plantenstoffen zijn wel belangrijk als verdedigingsmechanisme tegen belagers, zoals plantenetende insecten. Vanwege het economisch belang, zijn herbivore insecten en hun natuurlijke vijanden goed bestudeerd in cultuurgewassen. Echter, om een goed beeld te krijgen van de verdedigingsstrategieën die door planten worden gebruikt, is het belangrijk om ook te kijken naar deze strategieën in wilde soortgenoten van de gecultiveerde lijnen. Dit laatste is het hoofddoel van dit proefschrift.

Sommige herbivoren hebben speciale aanpassingen ontwikkeld om de negatieve effecten van secundaire plantenstoffen zoals GS te omzeilen. Rupsen van het grote en kleine koolwitje (*Pieris brassicae* en *P. rapae*), maar ook van de koolmot (*Plutella xylostella*) zijn zeer efficiënt in het onschadelijk maken van GS en lijken ogenschijnlijk niet negatief te worden beïnvloed door GS in hun voedsel. Bovengenoemde herbivoren eten alleen die plantensoorten die GS bevatten, voornamelijk in de Brassicaceae familie. Er zijn ook herbivoren die eten van plantensoorten in verschillende families, zogenaamde generalisten. Rupsen van de kooluil (*Mamestra brassicae*) eten niet alleen kool maar ook sla- en uienplanten. Deze laatste twee plantensoorten bevatten geen GS en behoren ook niet tot de Brassicaceae. Ook voor sluipwespen geldt dat verschillende soorten zich in meerdere of mindere mate gespecialiseerd hebben om alleen in bepaalde insectensoorten hun eitjes te leggen. In dit proefschrift, heb ik onderzoek gedaan naar de ontwikkeling van verschillende vlindersoorten

die zich wel of niet gespecialiseerd hebben op het eten van soorten in de Brassicaceae. De sluipwespen die ik heb gebruikt, varieerden in gastheerspecialisatie. Mijn verwachting was dat de ontwikkeling van zowel de herbivoren als hun sluipwespen negatief worden beïnvloed door kruisbloemige voedselplanten met hoge gehalten aan GS en dat generalisten sterker worden beïnvloed dan specialisten.

Effect van voedselplant op de ontwikkeling van herbivoren en hun sluipwespen

In hoofdstukken 3-7 presenteer ik mijn onderzoek naar de ontwikkeling van verschillende vlindersoorten en hun sluipwespen op gecultiveerde en wilde soortgenoten van spruitkool (*B. oleracea*) en een aantal mosterdsoorten (*B. nigra*, *B. juncea* en *Sinapis arvensis*, *S. alba*). De ontwikkeling van de herbivore specialisten (*Pieris brassicae* en *P. xylostella*) werd slechts in beperkte mate beïnvloed door variatie in voedselkwaliteit tussen de verschillende mosterdsoorten, ondanks de relatief hoge GS concentraties in hun bladeren. Dit laatste geldt voor zowel de cultivars als hun wilde soortgenoten. Verschillen in ontwikkeling hadden betrekking op de ontwikkelingsduur (van ei tot pop of van ei tot vlinder/mot en popgewicht/adultgewicht), terwijl de overleving hoog was op alle plantensoorten.



Hoewel de mosterdsoorten in dit onderzoek gekenmerkt worden door verschillen in GS samenstelling, lijken deze stoffen de groei van de specialistischerupsen niet negatief te beïnvloeden. De groei van de sluipwespen *Cotesia glomerata* en *Diadegma semiclausum*, die zich ontwikkelen in rupsen van respectievelijk *P. brassicae* en *P. xylostella*, werden nog minder dan hun gastheren beïnvloed door verschillen in voedselkwaliteit. Het efficiënte GS detoxificatie mechanisme in de gastheer voorkomt mogelijk dat de larven van de sluipwespen worden blootgesteld aan (hoge) GS concentraties. Een alternatieve verklaring is dat sluipwespen van gastheren die alleen kruisbloemigen eten een eigen detoxificatie systeem hebben ontwikkeld.

De wilde variant van de verschillende koolcultivars groeit langs de Atlantische kust van West-Europa. In hoofdstukken 4 en 5 heb ik gebruik gemaakt van een aantal populaties uit Engeland waarvoor al eerder is aangetoond dat ze aanzienlijk variëren in GS gehalten in de bladeren. Zaden van deze wilde populaties zijn gebruikt voor experimenten in een kas. De gecultiveerde spruitkoolvariëteit was kwalitatief een betere voedselplant voor de specialisten *P. rapae* en *P. xylostella* dan de wilde kool uit

Engeland. Net als voor de mosterdplanten, hadden verschillen in ontwikkeling van rupsen alleen betrekking op ontwikkelingsduur en pop/adult gewicht. De ontwikkeling van de sluipwespen *C. rubecula* en *D. semiclausum* weerspiegelde dat van hun respectievelijke gastheer, *P. rapae* en *P. xylostella*.

Naast de ontwikkeling van specialisten heb ik ook de ontwikkeling van generalisten op wilde en gecultiveerde kool vergeleken. De effecten van verschillen in voedselkwaliteit op de ontwikkeling van rupsen van de kooluil (*M. brassicae*) waren zeer groot. Rupsen ontwikkelden zich het snelst tot pop en bereikten tevens een hoger popgewicht op de spruitkool cultivar en één van de wilde populaties ('Kimmeridge') in vergelijking tot de wilde 'Old Harry' populatie. Op de derde wilde populatie bereikte echter geen enkele rups het popstadium 'Winspit'. Net als voor de specialisten, weerspiegelde het effect van de koolpopulatie op de ontwikkeling van de sluipwesp *Microplitis mediator* in *M. brassicae* rupsen, dat van de ontwikkeling van de gastheer.



Analyse van GS in de bladeren van de wilde koolplanten bracht grote verschillen in GS concentraties tussen de populaties aan het licht. Verder bleek dat de concentraties van bepaalde GS in de wilde populaties sterk toenamen nadat van de plant gegeten was, terwijl dat in veel mindere mate het geval was in de cultivar. Totale gehalten aan GS in aangevreten planten waren 2-4 keer zo hoog in de wilde populaties als in de cultivar. Met behulp van lineaire regressie heb ik een verband kunnen aantonen tussen de overleving van de generalist *M. brassicae* en concentraties van de glucosinolaten sinigrine, gluconapin en de totale hoeveelheid GS. Voor de specialist, *P. rapae* was er alleen een verband tussen ontwikkelingsduur van ei tot pop en neoglucobrassicine concentraties in de bladeren.

In hoofdstuk 4 beschrijf ik het onderzoek aan de ontwikkeling van de sluipwesp *Diadegma fenestrale* in *P. xylostella* rupsen op wilde en gecultiveerde kool en zwarte mosterd (*B. nigra*). Deze sluipwespsoort is nauw verwant aan *D. semiclausum*, maar kan zich niet alleen succesvol ontwikkelen in *P. xylostella* maar ook in een paar andere rupsensoorten. In tegenstelling tot *D. semiclausum* werd de ontwikkeling van *D. fenestrale* sterk beïnvloed door de voedselplant van *P. xylostella*. De overleving was hoger op spruitkool dan op de wilde 'Old Harry' populatie. De overleving was echter het laagst (slechts 5% van de eitjes ontwikkelde zich tot volwassen sluipwesp) als de gastheer van *B. nigra* at, de plantensoort die de zwaarste motten voortbracht.

De groei van herbivoren die zich gespecialiseerd hebben op kruisbloemige plantensoorten kan dus beïnvloed worden door verschillen in kwaliteit tussen plantensoorten maar



ook binnen een plantensoort. De effecten van voedselkwaliteit op specialisten hebben echter voornamelijk betrekking op de ontwikkelingsduur en biomassa van de pop/adult. Hetzelfde kan gezegd worden over sluipwespen die zich gespecialiseerd hebben op gastheren die voornamelijk van plantensoorten eten in de Brassicaceae familie. Met betrekking tot de generalist *M. brassicae* en de sluipwesp *M. mediator* had de voedselkwaliteit van wilde en gecultiveerde kool niet alleen invloed op de ontwikkelingsduur en biomassa, maar vooral ook op de overleving. De ontwikkeling van *M. brassicae* rupsen was duidelijk gerelateerd aan GS concentraties in de voedselplant. Dit in tegenstelling tot de specialisten waar de relatie tussen GS en de ontwikkeling van de rupsen afwezig of niet erg sterk was.

Is er een conflict tussen directe en indirecte verdediging?

Zoals eerder genoemd, kunnen planten zowel directe als indirecte verdedigingsmechanismen inzetten om schade door herbivoren te voorkomen of te reduceren. Echter, zoals ik ook heb laten zien, kunnen secundaire plantenstoffen niet alleen de ontwikkeling van herbivoren nadelig beïnvloeden maar ook die van sluipwespen die zich in of op deze herbivoren ontwikkelen. Er kan dus een conflict ontstaan tussen directe en indirecte plantenverdediging als een sluipwespvrouwje wordt aangetrokken naar een plant die niet zo goed is voor de ontwikkeling van haar nakomelingen. Dit is onderzocht in hoofdstuk 6 en 7. In deze studies heb ik de ontwikkeling van herbivoren en hun sluipwespen (alleen specialisten) gevolgd op verschillende mosterdsoorten (*B. nigra*, *B. juncea* en *S. arvensis*). In hoofdstuk 7 heb ik ook gekeken naar variatie binnen een plantensoort (*B. nigra*). Daarnaast heb ik bestudeerd of dezelfde plantensoorten en populaties verschillen in aantrekkelijkheid voor sluipwespvrouwjes. Zoals eerder gezegd waren de verschillen in ontwikkeling van de herbivoren en hun sluipwespen op de diverse mosterdsoorten niet erg groot. De sluipwespen (*D. semiclausum* en *C. glomerata*) hadden een voorkeur om te landen op planten die geen negatieve invloed hadden op de ontwikkeling van hun nakomelingen. Voor de plantensoorten die ik hier heb getest was er dus geen conflict tussen directe en indirecte plantenverdediging.

Plantenverdediging in gecultiveerde and wilde soortgenoten

Plantenveredeling is gericht op het versterken van bepaalde planteneigenschappen, zoals het vergroten van bepaalde plantendelen of het produceren van meer zaden, maar ook het

veranderen van chemische eigenschappen. Echter, sommige van deze kunstmatige veranderingen kunnen in conflict zijn met verdedigingseigenschappen zoals die aanwezig waren in de voorouder van het cultuurgewas. Als gevolg van deze selectie, kunnen concentraties van primaire en secundaire plantenstoffen dusdanig zijn gewijzigd dat deze planten meer vatbaar zijn voor bijvoorbeeld plantenetende insecten dan de wilde variant. In dit proefschrift heb ik laten zien dat veredeling (al dan niet bewust) van spruitkool geleid heeft tot een verlaging van GS concentraties in de bladeren en dat de herbivoren beter groeien op de cultivar dan op sommige wilde populaties. In de mosterdsoorten waren de GS concentraties relatief hoog, zowel in gecultiveerde variëteiten als in wilde populaties, terwijl de effecten daarvan op de ontwikkeling van de specialistische herbivoren gering waren. Het effect van plantenveredeling op eigenschappen die de vatbaarheid voor plantenetende insecten kunnen verhogen, hangt dus af van de eigenschap waarop is geselecteerd. In kool heeft veredeling geleid tot lagere GS concentraties in de bladeren terwijl selectie op de zaden in mosterdplanten de GS gehalten in de bladeren nagenoeg niet heeft veranderd. Het is onduidelijk in hoeverre veredeling in *B. nigra* heeft geleid tot veranderingen in de productie van plantengeuren die aantrekkelijk zijn voor sluipwespen. Conflicten tussen directe en indirecte verdediging in landbouwsystemen zullen niet zo snel optreden aangezien directe (chemische) verdedigingsmechanismen in cultuurgewassen vaak zijn verminderd.

Zoekgedrag van sluipwespen buiten de Y-buis en de windtunnel

In veel wetenschappelijk onderzoek gericht op het ontrafelen van plantengeuren die een rol spelen bij de aantrekking van sluipwespen wordt gebruik gemaakt van windtunnel en Y-buis opstellingen. In deze opstellingen krijgen sluipwespvrouwtjes de mogelijkheid een keuze te maken tussen (meestal twee) verschillende geurbronnen. Echter, in hun natuurlijke leefomgeving moeten sluipwespen hun gastheer zien te vinden in een complexe vegetatie bestaande uit meerdere plantensoorten die vaak zijn aangetast door verschillende herbivoren. In het laatste hoofdstuk heb ik het zoekgedrag van de sluipwesp *D. semiclausum* onderzocht in een iets meer natuurlijke opzet in vergelijking tot een windtunnel of Y-buis. De basisopstelling bestond uit een groep koolplanten waarvan alleen de middelste plant gastheren bevatte. Ik



heb vervolgens het gedrag van individuele sluipwespvrouwtjes gevolgd totdat zij in vijf rupsen eitjes had gelegd. Daarnaast heb ik gekeken of sluipwespvrouwtjes meer of minder efficiënt zijn in het vinden van de gastheren als er een niet-kruisbloemige plantensoort (gerst, *Hordeum vulgare*) of een andere kruisbloemige soort (witte mosterd, *S. alba*) aan de opstelling wordt toegevoegd. Uit de gegevens bleek dat *D. semiclausum* vrouwtjes meer moeite hebben met het vinden van de koolplant als er ook witte mosterd dan wel gerst in de opstelling aanwezig is. Blijkbaar interfereren de geuren die verspreid worden door de mosterdplanten met het zoekgedrag van de sluipwespen. Eerder onderzoek heeft aangetoond dat geuren van onbeschadigde witte-mosterdplanten net zo aantrekkelijk zijn als geuren van koolplanten die aangevreten zijn door hun gastheer. Gerstplanten lijken een fysiek obstakel te vormen die het vinden van de koolplant met gastheren belemmeren. Deze resultaten geven aan dat voorzichtigheid geboden is met betrekking tot het vertalen van voorkeuren van sluipwespen voor bepaalde geuren in windtunnels en Y-buizen naar het gedrag van sluipwespen in een natuurlijke vegetatie.

Het vergelijken van het zoekgedrag van sluipwespen en de populatie dynamica van de gastheer en de sluipwesp in gecontroleerde landbouwsystemen en in ongecontroleerde ecosystemen zullen nieuwe inzichten geven met betrekking tot de mechanismen die ten grondslag liggen aan tritrofe interacties. Daarnaast helpen zulke studies om het relatieve belang van bottom-up (plant-gerelateerde) en top-down (predatie/sluipwesp-gerelateerde) regulatie van geleedpotige herbivoren te openbaren.

DANKWOORD

Een proefschrift zonder dankwoord is als een huis zonder dak. Bij deze plaats ik dan ook het dak erop. Dit boek was er niet gekomen zonder Jeff Harvey (NIOO Heteren), mijn levenspartner maar tevens ook degene die mij heeft overgehaald uiteindelijk toch te promoveren. Jeff, I would like to thank you for your love, scientific input, but also for your assistance in some of the experiments. Who else would be willing to spend the weekend in a cold room at 4 °C? Having a partner like you makes it also easier to convert the bathroom into a climate room and the refrigerator into a storage cabinet for insects.

Natuurlijk ben ik ook dank verschuldigd aan mijn promotor Marcel Dicke. Jij hebt me de mogelijk geboden om dit onderzoek te doen. Ondanks het feit dat je tijdens de afronding van dit boek in China was, heeft dat de voltooiing op geen enkele wijze in de weg gestaan. Het contact met jou was intensiever vanuit China dan hier bij Entomologie in Wageningen. Ik heb grote bewondering voor de snelle en kritische respons op voorlopige versies van de diverse hoofdstukken.

Insecten en planten vormen de basis van dit onderzoek. Ik wil hierbij Leo Koopman, André Gidding en Frans van Aggelen bedanken voor hun inspanningen m.b.t. tot de continue aanvoer van sluipwespen en rupsen. Medewerkers van Unifarm dank ik voor het opkweken van de planten. In het bijzonder wil ik hier Bert Essenstam noemen die de meeste planten heeft opgekweekt, altijd van uitstekende kwaliteit.

Daarnaast wil ik Tibor Bukovinszky bedanken voor de prettige samenwerking, maar soms ook heftige discussies. Onze samenwerking heeft geleid tot experimenten die niet altijd even eenvoudig waren van opzet. Ik geef toe dat ik tijdens de eindeloze observaties van de sluipwespen in de tent, ze wel eens 'zag vliegen'. Gelukkig, heeft al dat getuur naar koolplanten en wespen twee mooie publicaties opgeleverd. Ik hoop van harte dat er meer zullen volgen. Verder is je inzet bij het parasiteren en je statistische kennis een grote hulp geweest. Dit geldt ook voor de statistische input van Lia Hemerik (Biometris) wat betreft de 'survival analyses' van de gedragsobservaties in hoofdstuk 8.

Hoewel het werk voornamelijk is verricht in Wageningen, hebben behalve Jeff ook diverse andere mensen van het NIOO (Dept. Multitrofe Interacties) in Heteren een belangrijke bijdrage geleverd aan dit onderzoek. Allereerst is daar natuurlijk Nicole van Dam. Jouw expertise op het gebied van glucosinolaten was en is onontbeerlijk. Daarbij word je technisch bijgestaan door Ciska Raaijmakers. Ciska, bedankt dat je mij op zo'n prettige wijze hebt ingewijd in de geheimen van de glucosinolaatextractie. Als ik weer eens in het lab op het NIOO aan het werk was, verschenen er ongemerkt allerlei zoetigheden en andere versnaperingen (Roxina Soler). En bijna was de vakantie naar Equador en de Galapagos niet doorgegaan, als Roel Wagenaar er niet geweest was om een van de experimenten af te ronden. Roel, bedankt daarvoor. Ik wil ook graag Wim van der Putten noemen, het grote multitrofische opperhoofd die dit alles heeft toegestaan. Wim, je bent ook degene geweest die Jeff heeft gewezen op het bestaan van de wilde populaties in Engeland. Dat was een erg goede zet die geleid heeft tot een vruchtbare samenwerking met James Bullock (Centre for Ecology and Hydrology, Oxford, England). James, I would like to thank you for introducing Jeff and me to the wild cabbage populations. Your hospitality and the time you spent driving us around are very much appreciated. I hope that the collaboration with you and Matthias Wichmann will continue.

Ik ben geen chemicus, maar gelukkig zijn Maarten Posthumus (Organische Chemie WU) en Kees Hordijk (NIOO, Nieuwersluis) dat wel. Ik wil jullie hierbij bedanken voor het analyseren

van de monsters met vluchtige stoffen.

Verder wil ik alle andere collega's van Entomologie bedanken die ik hier niet bij naam genoemd heb, maar die er wel voor zorgen dat de sfeer op heb lab zo plezierig is. En verder wens ik iedereen net zo'n prettige kamergenoot toe als ik heb in Jeroen Spitzen.

De boog kan niet altijd gespannen zijn. Vrienden zijn daarvoor zeer belangrijk. Ook al is het contact niet meer zo intensief als vroeger, ik wil hierbij Mieke, Johan, Lucienne en Jouke bedanken voor hun vriendschap.

Over ontspannen gesproken, Leo bedankt voor de liters koffie en cola. En ik hoop dat onze muzikale uitspattingen gewoon worden voortgezet.

Het oog wil ook wat hebben en dit boekje had er niet zo mooi uitgezien zonder de enorme inzet van Tibor (buitenkant en foto's) en Nina Fatouros (opmaak binnenkant). Heel erg bedankt!

Als laatste wil ik Ina Edens noemen, het allerliefste zusje van de hele wereld. Fijn dat je me 21 mei moreel komt ondersteunen.

Rieta

Wageningen, Maart 2008

CURRICULUM VITAE

Rieta Gols was born on March 19 1965, in Veendam, in the Netherlands. She completed secondary education (V.W.O.) in Alkmaar in 1983 and finished technician school (Hoger Laboratorium Onderwijs) in Amsterdam in 1987. In 1988, she got her first year degree at Wageningen University (then Agricultural University). From 1989 until 1992, she worked at a contract laboratory (Notox bv) where she was responsible for aquatic toxicity tests with fish and algae. In April 1992, she began working as a technician at the Laboratory of Entomology at Wageningen University and was involved in several projects such as the development of antifeedants against insect herbivores and tritrophic interactions among plants, spider mites and predatory mites. In 2000, she took up a similar position at the Free University in Amsterdam where she studied the behavior of springtails in a molecular framework. Returning to Wageningen in 2002, she began to study herbivore and parasitoid development and foraging behaviour. She was also involved in the discovery of a pathogen in an important biological control agent. When in 2004 she was introduced to the 'wild' cabbage populations growing along the south coast of Dorset, England, she finally decided to commence a PhD project. She will continue studying tritrophic interactions on these plants at the Laboratory of Entomology in Wageningen.



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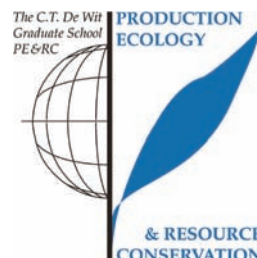
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- Gols R**, Harvey JA. Plant mediated effects in the Brassicaceae on the performance and behaviour of parasitoids.
- Gols R**, van Dam NM, Raaijmakers CE, Dicke M, Harvey JA. Comparing direct and indirect plant defences in *Brassica nigra* populations that have been exposed to different selection pressures.

PE&RC PhD Education Certificate

With the educational activities listed below the PhD candidate has complied with the educational requirements set by the C.T. de Wit Graduate School for Production Ecology and Resource Conservation (PE&RC) which comprises of a minimum total of 32 ECTS (= 22 weeks of activities)



Review of Literature (5.6 ECTS)

- Plant mediated effects in the Brassicaceae on the behaviour and performance of parasitoids and predators (2008)

Writing of Project Proposal (7 ECTS)

- Linking direct and indirect plant defences in wild and cultivated Crucifers (2005)

Post-Graduate Courses (5.5 ECTS)

- Spring school: chemical communication: from gene to ecosystem; PE&RC, EPS (2005)
- 4th PhD Symposium on “The Exploration of Ecological Interactions with Molecular and Chemical Techniques”; Int. Max Planck Res. School, EPS (2006)
- Advanced statistics; PE&RC (2006)
- Multivariate analysis; PE&RC (2007)

Deficiency, Refresh, Brush-up Courses (2.6 ECTS)

- X-calibur software: analysis GC-MS data; Interscience, Breda (2004)
- Basic statistics; PE&RC (2006)

Competence Strengthening / Skills Courses (1.7 ECTS)

- Scientific writing; WGS (2006)
- Presentation skills; Centa, WU (2006)

Discussion Groups / Local Seminars and Other Scientific Meetings (6.6 ECTS)

- VICI group and Crufer Group meetings (2004-2008)
- Entomology PhD discussion group (2006-2008)

PE&RC Annual Meetings, Seminars and the PE&RC Weekend (1.2 ECTS)

- PE&RC day: the truth of science (2005)
- PE&RC 10 year anniversary (2005)
- Workshop on plant-insect interactions: from molecular biology to ecology (2006)
- PE&RC day: who pulls the strings? (2006)

International Symposia, Workshops and Conferences (12 ECTS)

- 6th Int. Seminar on Apterygota; Siena University Italy (2002)
- IX European workshop on insect parasitoids; Cardiff, UK (2005)
- ESF-VOCBAS: improving the interpretation and application of plant volatile analysis; Wageningen (2006)
- First international conference on glucosinolates; Jena, Germany (2006)
- SIP 13: 13th Symposium on insect-plant relationships; Uppsala, Sweden (2007)

Courses in which the PhD Candidate Has Worked as a Teacher

- Ecology: holly leaf miner practical course; Entomology, 3 days/year (2005-2008)

Thesis layout: Nina Fatouros, Bugsinthepicture.com Visual Studios, The Netherlands.

Cover design: Tibor Bukovinszky, Bugsinthepicture.com Visual Studios, The Netherlands.

Photography: Tibor Bukovinszky, Hans Smid (both Bugsinthepicture.com Visual Studios, The Netherlands), Rieta Gols & Caroline Muller.

Printed by Print Partners Ipskamp, Enschede, The Netherlands.