Plant-herbivore interactions in natural *Brassica oleracea* communities

Submitted by:

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To the University of Exeter as a thesis for the degree of
Doctor of Philosophy

April 2009

I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

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Abstract

Co-evolutionary interactions between plants and herbivores are suggested to be the driving force behind the high diversity observed in plant secondary metabolites. These compounds play an important role in herbivore resistance mechanisms in many plant species. An individual plant can produce and store a number of structurally different secondary compounds. Variation in plant chemical profiles is commonly observed within and between natural populations across a wide range of taxa, yet the ecological importance of this variation is still a major question in the area of plant-herbivore interactions.

In this thesis I use wild cabbage (*Brassica oleracea* var. oleracea) plants in twelve naturally established populations to investigate plant-herbivore interactions mediated by structural variation in aliphatic glucosinolates, a class of secondary metabolites produced by the *Brassicaceae*.

Overall, the results showed that several herbivore species respond to the genetically determined variation in glucosinolate profile, indicating that the structure of the local herbivore community can be influenced by variation in plant defence chemistry. In addition, the direction of herbivore responses to different plant chemical phenotypes differed between species. A finer scale study which focused on the interactions between an herbivore and aliphatic glucosinolate variation supported the general trend observed in the large scale study. Glucosinolate profile was also found to have an impact on plant seed set.

The findings show that glucosinolate profiles may be under selection in these natural plant populations and provide some support for the role of herbivores in the maintenance of secondary metabolite diversity.

Acknowledgements

Firstly, I'd like to thank my supervisors Dave Hodgson and James Bullock for giving me the opportunity to work on a PhD project which I have thoroughly enjoyed. Dave's incredibly supportive, approachable attitude and unfailing enthusiasm for the project have made the past few years a steep but very rewarding learning curve for me. James I thank for always making the time to respond quickly with helpful comments whenever I sent him thesis chapters or paper manuscripts, and for introducing me to Matthias Wichmann, who was a great help in selecting field sites in Dorset, and Nicole van Dam, who provided email support when I started carrying out the HPLC analysis. Jon Blount was also very helpful whenever technical problems with the HPLC equipment arose. Thanks also to two undergraduate project students, Martin Durman and Rosie Daines for fieldwork assistance.

Many thanks to the Hodgson research group for all their feedback and support, in particular ex-EXPECT team members Mairead Maclean and David Carslake. Many other friends at Tremough have been instrumental in making the last three years a really enjoyable experience and I would particularly like to thank Michelle Taylor, Sahran Higgins, Wiebke Schuett, Ruth Leeney, Lorna Shaw, Mel Smee, Lucy Wright, Stine Turner and James Grecian.

Isobel and the rest of the Giblin family I cannot thank enough for not only keeping an eye out for cabbages on the Devon coast around their house and reconnoitring a field site for me, but also for their amazing hospitality while I carried out fieldwork in Devon. I'm also very thankful for Isobel's company and help (and of course Poppy the dog's!) on some of my first field visits.

Finally, I owe so many thanks to my mum, Michéle, for all her encouragement and support.

Contents

List of Figures

Chapter 1:

Chapter 2:

Chapter 3:

Chapter 4:

Chapter 5:

Chapter 6:

List of Tables

Chapter 2:

Chapter 3:

Author's declaration

Statement of contribution to co-authored papers:

I carried out all data collection for each of the chapters, including herbivore surveys, seed collection and HPLC analysis of glucosinolates. I carried out all statistical analysis under the guidance of Dave Hodgson and James Bullock and I followed a methodology developed by Dave Hodgson for the spatial inter-population statistical analysis (Chapters 2 and 3). I initially drafted all sections of the chapters and amended later drafts following the advice of D. J. Hodgson, J. M. Bullock and, for Chapters 2 and 4, several anonymous journal reviewers.

Chapter 1

Ecological mechanisms maintaining natural variation in secondary metabolites.

Secondary metabolites are organic compounds present in all higher plants (Wink 2003). While basic metabolism refers to the processes crucial for cell maintenance and development, secondary metabolites are not necessary for the survival of the cell *per se*, but are required for the survival of the cell in its natural environment (Kliebenstein 2004). Major functions of secondary metabolites include chemical defence and signalling, deterring herbivore attack, and attracting pollinators or seed dispersers (Wink 2003). These compounds are required for a wide range of biological roles and are closely associated with plant fitness (Kliebenstein 2004).

High levels of qualitative and quantitative variation are observed within many classes of secondary metabolite across a large number of plant families (Wink 2003) . An individual plant may produce and accumulate several compounds that show slight variation in structure (Kliebenstein 2004). However, the biological activity of this secondary metabolite diversity is still poorly understood (Macel et al. 2002; Kliebenstein 2004) and questions about the ecological importance of each compound and the impact of secondary metabolite variation on plant fitness and environmental interactions remain unanswered (Kliebenstein 2004).

The hypothesis that herbaceous plants produce secondary metabolites for defence against herbivores is a long standing one (Fraenkel 1959). Coevolutionary arms races between plants and herbivores are thought to be the driving force behind the evolution of the extensive structural diversity observed within secondary metabolite classes (Ehrlich and Raven 1964): selection favours plants that produce novel defence compounds when herbivores have developed resistance mechanisms to previous compounds. Furthermore, insect species experience selection to become more

specialised on a particular class of secondary metabolites as they develop physiological mechanisms to detoxify the novel defence compounds in their food plant (Ehrlich and Raven 1964).

Regardless of the mechanisms originally responsible for driving the diversification of plant defence compounds, if the production of these compounds is costly then evolutionary theory would predict that the optimum compound or group of compounds would spread to fixation. But instead, widespread inter- and intra-population variation in secondary metabolite structure and concentration is observed across many plant species and groups of secondary metabolites including glucosinolates in Brassicaceae (Mithen et al. 1995b; Moyes et al. 2000), furanocoumarins in Apiaceae and Rutaceae (Berenbaum 1978; Berenbaum et al. 1986), and cyanogenic glycosides in Fabaceae (Kakes and Chardonnens 2000; Richards and Fletcher 2002).

MECHANISMS FOR THE MAINTENANCE OF SECONDARY METABOLITE DIVERSITY

Three ecological mechanisms can explain the maintenance of the observed levels of heritable diversity in secondary plant compounds in natural populations. First, as secondary metabolites are believed to have played a major role in the evolution of plant-insect interactions (Fraenkel 1959; Ehrlich and Raven 1964), differential selection by herbivores is a plausible explanation for the maintenance of this diversity. If a secondary metabolite confers an advantage in the presence of an herbivore species, but has a negative effect on plant fitness in the absence of the species or in the presence of an alternative species, fluctuations in the herbivore community could prevent a single genotype gaining a consistent advantage. Second, the abiotic environment is also a powerful selective pressure on plants and there is evidence for secondary metabolites playing a role in frost tolerance (Dirzo and Harper 1982) or being influenced by altitude (Richards and Fletcher 2002), demonstrating a potential for abiotic factors to maintain

diversity through bottom-up differential selection. Third, the selection for or against secondary metabolic variants may be weak, causing metabolic diversity to drift through ecological time and be maintained through mutation and pollen or seed transfer between plant populations. This hypothesis has strong links with the neutral theories of evolution (Kimura 1991) and biodiversity (Hubbell 2001).

EVIDENCE FOR THE ROLE OF HERBIVORES

There are four lines of evidence supporting the hypothesis that herbivores play a role in maintaining secondary metabolite variation: first, nearly all of the structural variation within classes of secondary metabolites is generated via modification of a side-chain on a backbone structure. This means that the basic biological action of the compound is not lost and novel side chains can be generated rapidly (potentially by a single mutation in a biosynthetic gene), conferring an advantage in an evolutionary arms race situation (Kliebenstein 2004). Second, in several secondary metabolite classes the compounds are stored in the plant as non-toxic stable compounds, with an enzyme that catalyses the formation of toxic products upon damage to the plant tissue: this binary system is ideally suited to herbivore defence systems as damage by herbivores initiates the immediate release of toxic compounds (Zagrobelny et al. 2004). Third, an individual plant may produce and store a wide range of secondary metabolites: a plant species will normally suffer attack from a number of different herbivore species and if the efficacy of individual compounds on herbivore species varies this may encourage chemical diversity (Feeny 1992). Fourth, specialist herbivores have evolved an equally wide range of detoxification mechanisms, for example two *Brassica* specialists have very different physiological resistance mechanisms; the diamondback moth (*Plutella xylostella*) produces a sulphatase enzyme to prevent the hydrolysis of glucosinolates, a class of secondary metabolites produced by the *Brassicaceae*, into toxic end products;

whereas the small white butterfly (*Pieris rapae*) redirects glucosinolate hydrolysis to form less toxic nitriles as an end product (Wittstock et al. 2004).

Secondary metabolites produced by host plants can have significant impacts on herbivore fitness, so can act as a strong selection pressure. These effects of plant defence compounds may be direct, for example, causing herbivore mortality or reducing growth rate (Li et al. 2000; Agrawal and Kurashige 2003). Alternatively, the actions of the secondary metabolites may be mediated by herbivore natural enemies: the hydrolysis of plant defence compounds in response to herbivore attack causes the emission of volatiles which can act as host location cues for parasitoids (De Moraes et al. 1998; Kessler and Baldwin 2001). Some of the most persuasive evidence for the maintenance of secondary metabolite diversity by top-down differential selection comes from studies showing species-specific responses of herbivores to plant defence compounds (Chew 1977; Huang and Renwick 1994; Linhart and Thompson 1995; Castellanos and Espinosa-Garcia 1997; Li et al. 2000; Harvey et al. 2007; Gols et al. 2008b). A major research theme for investigations into the effects of plant defence chemicals on herbivores has involved clarifying differences in the responses of generalist and specialist herbivores. Plant compounds that effectively deter generalist herbivore species often stimulate feeding or oviposition in species that have evolved mechanisms to avoid or detoxify plant defence chemicals (Berenbaum and Zangerl 1998; Musser et al. 2002). Furthermore, some specialists sequester plant chemicals, causing the effects of secondary metabolites to cascade up to higher trophic levels (Harvey et al. 2005). A common observation among the two feeding guilds is that generalists show a negative response to secondary metabolite concentration; whereas specialist herbivores are less affected by secondary metabolites, in some cases showing a positive response to secondary metabolite concentration, (Giamoustaris and Mithen 1995; Kliebenstein et al. 2002; Lankau 2007; Gols et al. 2008)

This evidence implies that herbivores respond to qualitative variation in plant secondary metabolites and the selection pressures imposed by herbivores may have differential effects on plant fitness. However, an infrequently addressed aspect of this topic is the ecological importance of the each secondary metabolite and what maintains the variation in secondary metabolite structure observed in natural populations. Studies on interactions between the wild parsnip (*Pastinaca sativa*) and its specialist herbivore, the parsnip webworm (*Depressaria pastinacella*) (Berenbaum 1978; Berenbaum et al. 1986; Berenbaum and Zangerl 1998), have shown that the webworm varied in its development rate when consuming structurally diverse secondary metabolites. Furthermore, the furanocoumarin profile of plants in different populations of wild parsnip closely matched the detoxification ability of parsnip webworms feeding on the population, indicating that the selection pressures exerted by the local herbivore population may be driving the diversity in chemical phenotypes. Conversely, Macel et al (2002) found no evidence that qualitative variation in pyrrolizidine alkaloids, secondary metabolites produced by *Senecio* species, had any effect on the cinnabar moth (*Tyria jacobaea*), and concluded that this specialist herbivore was unlikely to play a role in secondary metabolite diversity in this system.

In this thesis I investigated plant-herbivore interactions mediated by structural variation in secondary metabolites, and determined the potential for herbivore selection pressures to play a role in the maintenance of plant chemical diversity in natural populations of wild cabbage (*Brassica oleracea* var. oleracea).

STUDY SPECIES

Wild cabbage (*Brassica oleracea* L. var. oleracea) is a long lived, perennial member of the *Brassicaeceae* and is classed as a UK native (Preston et al. 2002). Natural populations of wild cabbage are confined to coastal cliffs, typically on chalk or

limestone substrata (Mitchell and Richards 1979). All members of the *Brassicaeceae* family produce glucosinolates, a highly diverse family of secondary metabolites (Fahey et al. 2001). Wild cabbage is genetically distinct from cultivated *Brassica* varieties (Preston et al. 2002) and contains higher glucosinolate concentrations (Gols et al. 2008).

A total of twelve wild cabbage populations across three counties in southwest England will be studied in the following chapters: three populations in Cornwall: PC1 (50°10'N, 5°42'W), PC2 (50°12'N, 5°42'W), and PC3 (50°10'N, 5°41'W), three in Devon: WAT (50°50'N, 3°51'W), WH (50°46'N, 3°49'W) and KW (50°34'N, 3°56'W) and six in Dorset: OH (50°64'N, 1°92'W), DD (50°62'N, 2°27'W), AH (50°69'N, 2°05'W), WS1 (50°59'N, 2°03'W), WS2 (50°58'N, 2°04'W) and K2 (50°60'N, 2°13'W). The locations of these populations are shown in figures 1.1-1.4.

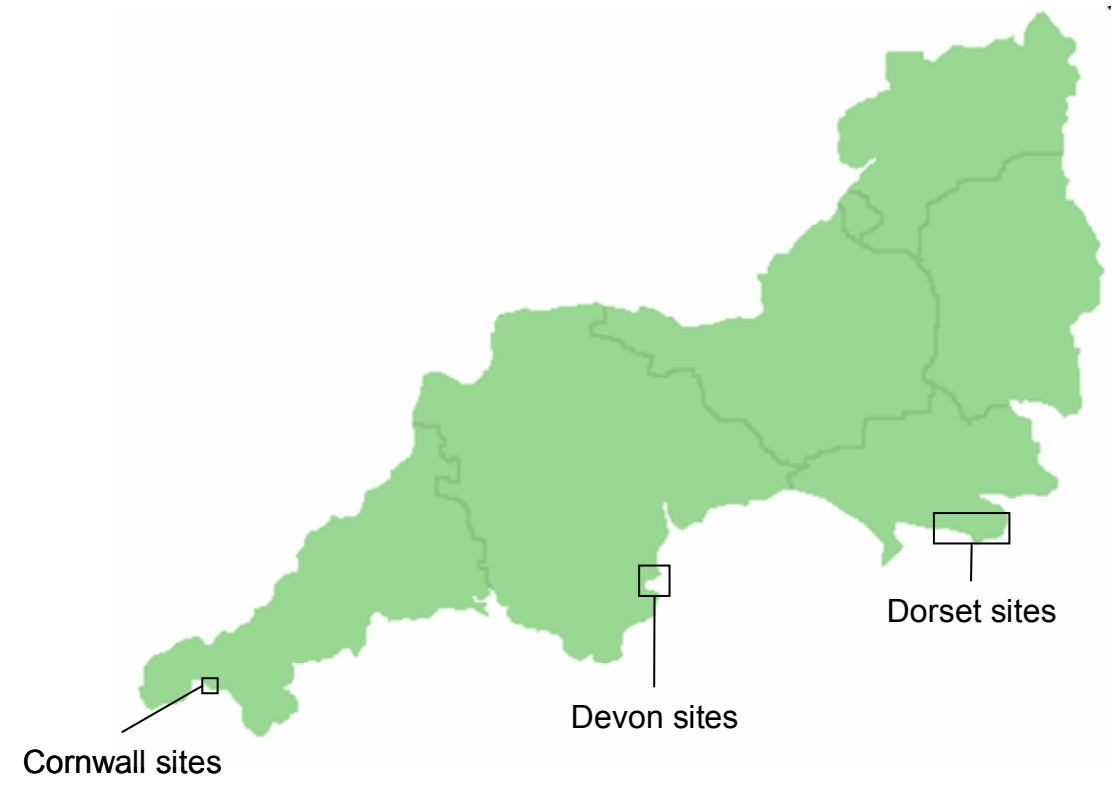

Figure 1.1. General location of field sites in south west England.

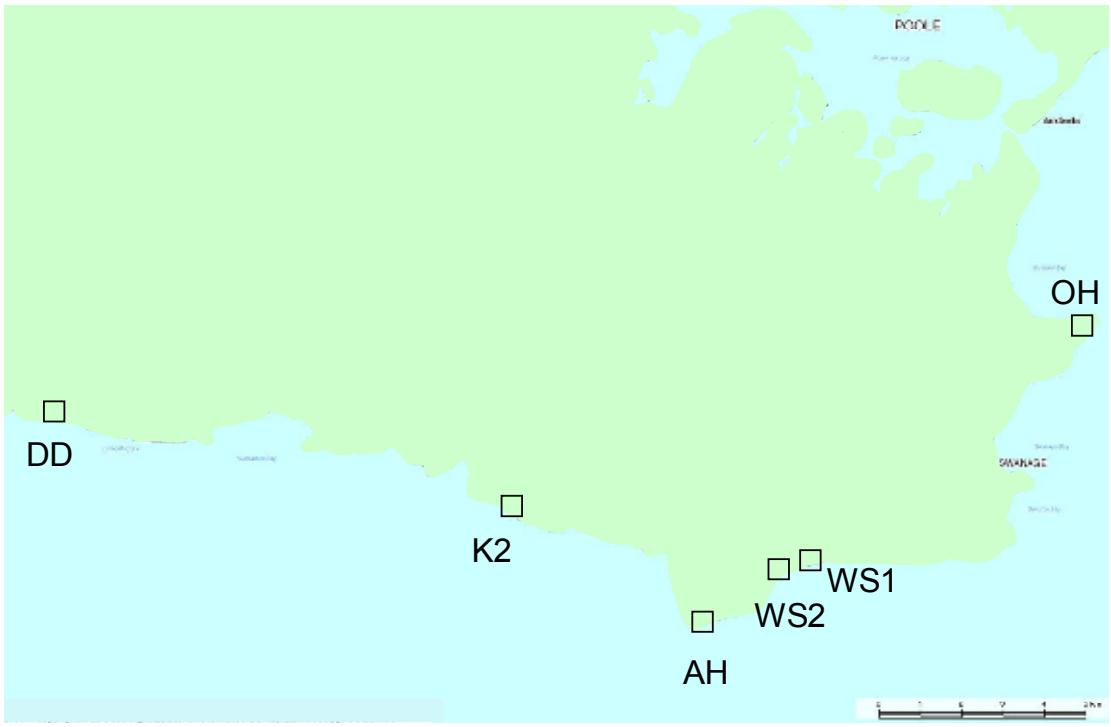

Figure 1.2. Locations of Dorset field sites

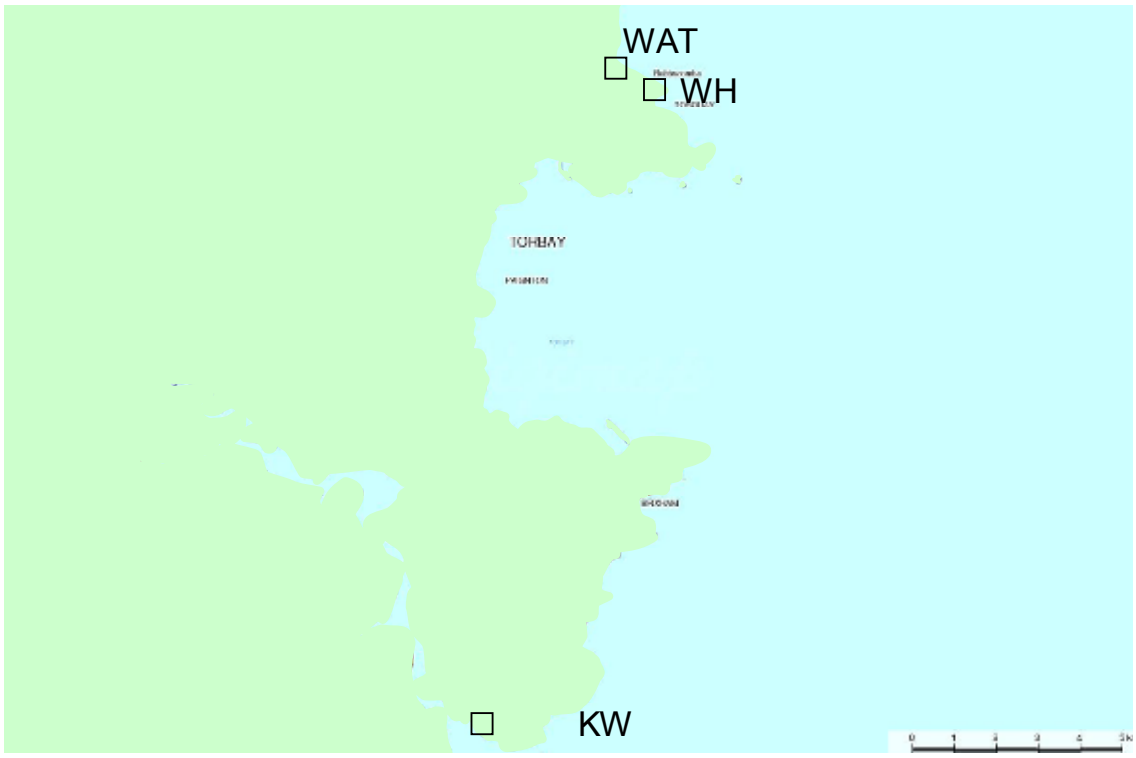

Figure 1.3. Locations of Devon field sites

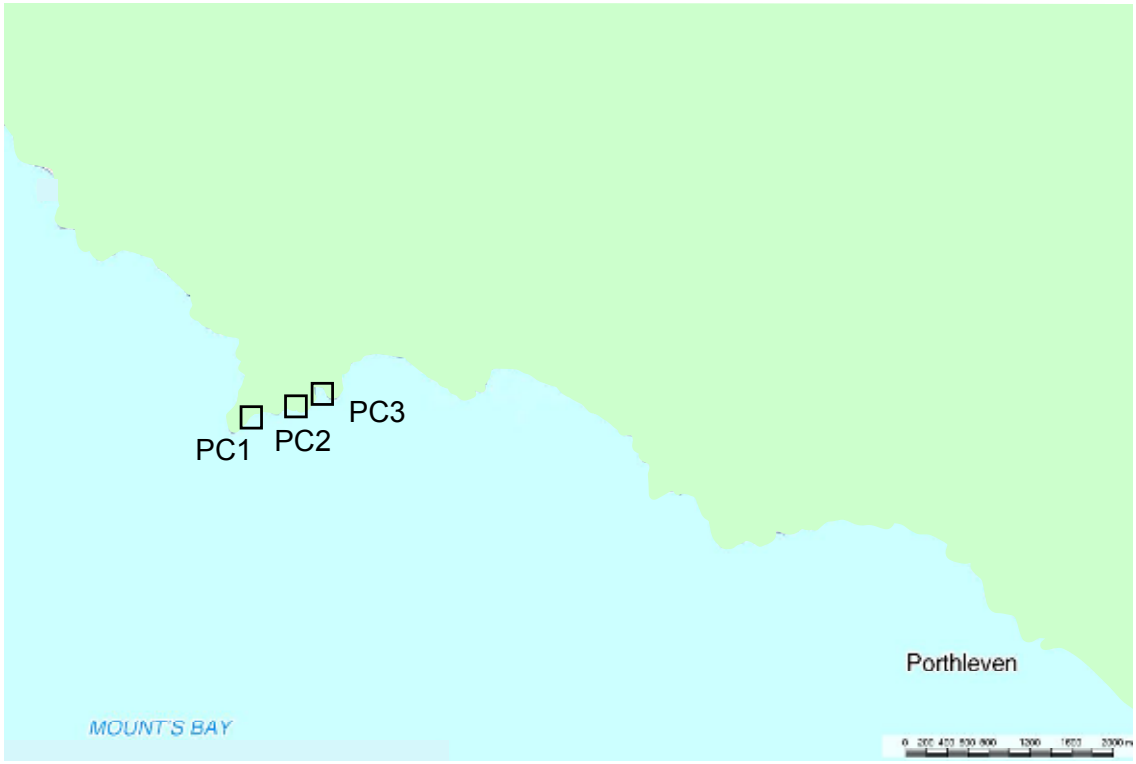

Figure 1.4. Locations of Cornwall field sites

All glucosinolates share a common backbone of a β-thioglucose linked to a sulphonated oxime group with a variable amino acid-derived side-chain (Fahey et al. 2001). Glucosinolates are divided into three classes depending on the amino acid used in the biosynthesis of the side-chain: aliphatic glucosinolates usually have a side-chain derived from methionine, but other straight chain amino acids such as alanine, leucine and isoleucine also occur (Fahey et al. 2001; Mithen 2001; Halkier and Gershenzon 2006). Aromatic glucosinolates are derived from phenylalanine and tyrosine, and indole glucosinolate are derived from tryptophan (Mithen et al. 1995a; Wittstock and Halkier 2002). The amino acid side-chains undergo extensive elongation and modification producing a diversity of glucosinolate structures (Halkier and Gershenzon 2006). The three structural classes are under varying levels of genetic control: indole glucosinolates are influenced by environmental factors (Martin and Muller 2007) and are under weak genetic control (Bodnaryk 1992; Rucker and Robbelen 1994; Bartlet et al. 1999), whereas the biosynthesis of aliphatic glucosinolates is under strong genetic control by five loci (Fig. 1.5), two loci: *GSL*-elong and *GSL-pro* control the length of the side-chain and three loci: *GSL-sulph, GSL-alk* and *GSL-oh,* control modification of the side-chain (Magrath et al. 1994; Parkin et al. 1994; Mithen et al. 1995a).

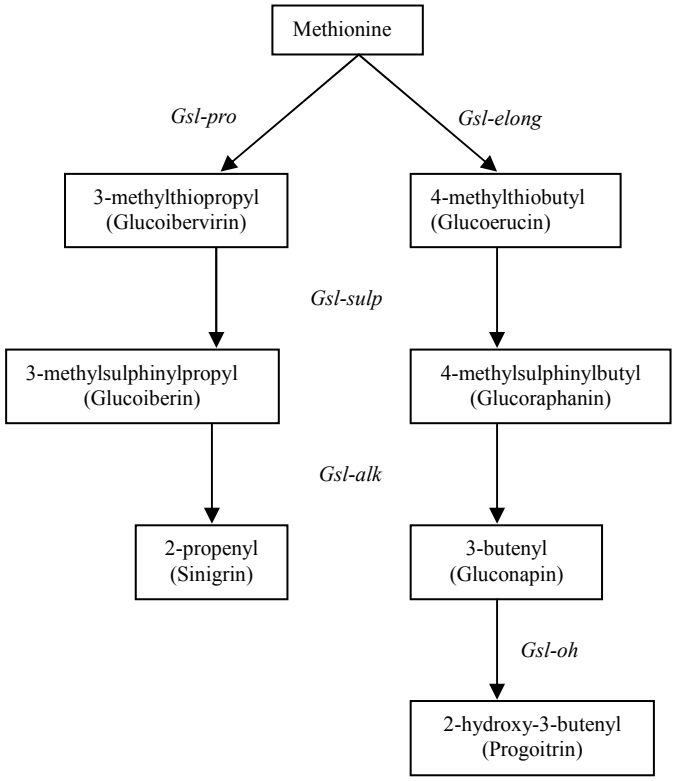

Figure 1.5. Diagram of aliphatic glucosinolate loci (adapted from Mithen et al., 1995a). Names of loci regulating side chain modification are shown in italics.

Previous studies have reported that the concentration of aliphatic glucosinolates in a plant are not affected by environmental factors (Giamoustaris and Mithen 1996; Bartlet et al. 1999). However, recent studies have found that aliphatic glucosinolate concentrations vary with herbivore damage (Soler et al. 2005) and even under controlled greenhouse conditions (Gols et al. 2007). Recent studies on *Arabidopsis* show that genes in the R2R3 MYB transcription factor family are strong candidates for regulatory genes controlling aliphatic glucosinolate biosynthesis (Gigolashvili et al. 2007; Sonderby et al. 2007; Beekwilder et al. 2008); damage to the plant causes temporary induction of these transcription factors (Beekwilder et al. 2008) which up-regulate the expression of aliphatic biosynthetic genes and increase the production of aliphatic glucosinolates in leaves (Sonderby et al. 2007). Although quantitative changes in glucosinolates occur, the qualitative biosynthesis of aliphatic glucosinolates is under strong genetic control (Magrath et al. 1994; Parkin et al. 1994; Mithen et al. 1995a) and therefore the individual aliphatic glucosinolates that a plant is able to synthesise will be consistent. For this reason, and because in this study samples for glucosinolates analysis were only collected once from each plant, the presence or absence of aliphatic glucosinolates will be considered.

IMPORTANCE OF FIELD STUDIES

There have been relatively few field based studies of glucosinolate mediated plant-herbivore interactions. In general, species-specific responses to variation in metabolites in natural situations have not been observed: although under some circumstances there is evidence for herbivore preferences to secondary plant compounds (Mithen et al. 1995b; Moyes et al. 2000; Moyes and Raybould 2001).

Two previous studies have investigated the variation in wild cabbage populations in Dorset, southwest England. A study by Mithen et al. (1995b) found

significant variation in presence of aliphatic glucosinolates. Isozyme analysis of the plants found that glucosinolate loci showed greater allelic variation compared to isozyme loci; indicating the variation in glucosinolate profiles is not due to founder effects or drift. Moyes et al (2000) also studied four wild cabbage populations in Dorset and found variation in presence or absence of individual glucosinolates. The authors found no link between plant glucosinolate profiles and herbivory by *Pieris* butterfly species, snails, slugs, flea beetles or aphids. However, one moth species (*Selania leplastriana*) showed a preference for plants containing higher concentrations of two glucosinolates.

Glucosinolate variation in wild cabbage is a useful system in which to investigate the role of herbivores in maintaining secondary metabolite variation, for a number of reasons. Plants in the Brassicaceae family are attacked by a wide variety of different herbivores, including generalists, specialists, phloem feeders, leaf chewers, leaf miners, root herbivores, and herbivores that feed on the reproductive parts of the plant. Many of these herbivores are economically important agricultural pests. But, while crop plants have been selectively bred and may not show the outcomes of plant-herbivore-natural enemy interactions, wild plants have not undergone artificial selection and therefore studies on wild species may elucidate glucosinolate-mediated interactions between plants and their herbivores that are no longer observed in crop varieties. Brassicas are the closest agricultural relatives of *Arabidopsis*; a model plant with a fully sequenced genome. *Arabidopsis* is also attacked by a number of pest species and the pathways of herbivore defence that are employed by *Arabidopsis* are widely conserved in many plant families (Mitchell-Olds 2001) so studies on *Arabidopsis* have provided relevant information on the genetics of defence chemistry in wild cabbage.

The overall aim of this thesis was to test hypotheses concerning plant-herbivore interactions that may promote the maintenance of secondary metabolite diversity by herbivore-mediated top-down differential selection. If herbivores play a role in the maintenance of glucosinolate profile diversity then we should observe:

1. Structuring of the herbivore community according to secondary metabolite variation.
2. Impacts of plant defence chemistry on herbivore fitness. The effects may be direct, or mediated by herbivore natural enemies.
3. Differential impacts of herbivores on the fitness of plants varying in chemical phenotype, shown by experiments carried out in natural populations.

In order to investigate these hypotheses and determine the potential for top-down differential selection by herbivores to promote variation in heritable variation in glucosinolate profile, I carried out surveys in 12 naturally established wild cabbage populations and in doing so highlighted the importance of population scale replication in plant ecology. I explored glucosinolate-mediated plant-herbivore interactions that have been investigated in laboratory-based studies in order to determine whether signals are identified in natural environmental settings, and used statistical tools for analyses of spatial non-independence. The specific aims of this thesis are as follows:

1. To investigate the magnitude of intra- and inter-population variation in aliphatic glucosinolate profiles across a large number of natural *B. oleracea* populations (Chapter 2)
2. To complete a three year survey of the herbivore community on plants of known glucosinolate profile in order to explore correlations between herbivore presence

and plant chemistry, and determine the potential for glucosinolates to structure the herbivore community (Chapter 2 and 3).

3. To carry out an intensive study on the interactions between plants, a specialist aphid and its natural enemies in order to investigate glucosinolate-mediated top-down and bottom-up effects on aphid colonies in natural populations (Chapter 4).
4. To use the variation in glucosinolate profiles occurring in natural populations and manipulate aphid colonies and their natural enemies to determine the effects on plant seed set (Chapter 5).
5. To investigate the broad-scale effects of glucosinolate profiles and herbivore presence on plant seed production across a large number of natural plant populations (Chapter 6).

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Chapter 2

Glucosinolate polymorphism in wild cabbage (*Brassica oleracea*) influences the structure of herbivore communities.

Published in Oecologia

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Abstract

Natural plant populations often show substantial heritable variation in chemical structure of secondary metabolites. Despite a great deal of evidence from laboratory studies that these chemicals influence herbivore behaviour and life history, there exists little evidence for the structuring of natural herbivore communities according to plant chemical profiles. *Brassica oleracea* (Brassicaceae) produces aliphatic glucosinolates which break down into toxins when leaf tissue is damaged. Structural diversity in these glucosinolates is heritable, and varies considerably at two ecological scales in the UK: both within and between populations. We surveyed herbivore attack on plants producing different glucosinolates, using 12 natural *B. oleracea* populations. In contrast to the results of previous studies in this system, which suffered low statistical power, we found significant differential responses of herbivore species to heritable glucosinolates both within and between plant populations. We found significant correlations between herbivore infestation rates and the presence or absence of two heritable glucosinolates: sinigrin and progoitrin. There was variation between herbivore species in the direction of the response and the ecological scale at which responses were identified. The correlations for some herbivore species changed at different times of the year. We conclude that variation in plant secondary metabolites can structure the community of herbivores that attack them, and propose that herbivore-mediated differential selection deserves further investigation as a mechanism maintaining the observed diversity of glucosinolates in wild *Brassica*.

Keywords: Aliphatic glucosinolate, *Brassica oleracea*, ecological scale, herbivory, plant-insect interaction.

Introduction

Plants produce a variety of secondary metabolites that increase their fitness by enabling them to cope with environmental stresses (Kliebenstein 2004). Many such compounds are widely regarded as important in herbivore defence (Mithen et al. 1995b). However, many plant species defy the simple evolutionary prediction that the most effective antiherbivore defence should spread to fixation in populations. Instead, we observe significant variation in heritable secondary metabolite profiles both within and between populations (Berenbaum et al. 1986; Harvey et al. 2005; Mithen et al. 1995b; Moyes et al. 2000; Richards and Fletcher 2002; van Leur et al. 2006). This raises two important questions. First, does variation in secondary metabolites result in the structuring of herbivore communities, mediated by coevolved preferences or aversions to specific chemicals (Bangert et al. 2006; Dungey et al. 2000; Maddox and Root 1990; Wimp et al. 2007)? Second, can this structuring of the herbivore community result in differential selection pressures that help to maintain secondary metabolite diversity (Lankau and Strauss 2008; Mithen et al. 1995b)? The first step towards answering these questions requires an analysis of correlations between plant secondary metabolite profiles, and the frequency and intensity of herbivory, in wild plant populations.

Debate surrounding the role of secondary metabolite variation in plant-herbivore interactions has been fuelled by studies of particular plant-herbivore systems, including cyanogenic glycosides in *Trifolium repens* (Fabaceae) (Hughes 1991) and furanocoumarins in *Pastinaca sativa* (Apiaceae) (Berenbaum 1978; Zangerl and Berenbaum 2003). A number of studies have clarified the effects of secondary metabolites on herbivore fitness (Agrawal 2000; Gols et al. 2008b; Lankau 2007; Li et al. 2000) and a common method used is the experimental induction of chemical defences to manipulate quantitative variation in plant chemistry (e.g. Agrawal (1999)).

In addition, there is evidence that when under attack by different herbivore species, plants show species-specific direct (e.g. Thaler et al 2001) and indirect (e.g. De Moraes et al 1998) induction of plant defences, with differential impacts on the structure of the herbivore community (Agrawal 2005).

Glucosinolates comprise a highly variable class of secondary metabolites found in the Brassicaceae plant family. Natural populations of wild cabbage (*Brassica oleracea* L. var. *oleracea*, subsequently referred to as *B. oleracea*) show significant inter- and intra-population variation in heritable aliphatic glucosinolates (Mithen et al. 1995b; Moyes et al. 2000) and offer an excellent model system to investigate secondary metabolite-mediated plant-insect interactions. Cultivated *B. oleracea* is a globally important crop and its genetics and biochemistry are well characterised (Gao et al. 2007; Magrath et al. 1994; Mithen et al. 1995a; Parkin et al. 1994). A wide variety of generalist and specialist herbivores attack wild cabbage and many of these herbivores are economically important *Brassica* crop pests. Wild cabbage is a perennial plant that exists in discrete coastal populations (Wichmann et al. 2008) allowing seasonal and inter-annual fluctuations in the herbivore community to be studied.

A number of laboratory assays investigating the responses of herbivores to *Brassica* secondary metabolites have revealed species-specific aversions and attractions to qualitative variation in glucosinolates (Castellanos and Espinosa-Garcia 1997; Chew 1977; Gols et al. 2008b; Harvey et al. 2007; Huang and Renwick 1994; Li et al. 2000; Linhart and Thompson 1995). Work carried out on sinigrin glucosinolate in *Brassica nigra* shows that generalist and specialist herbivores exhibit different responses to sinigrin and suggests that heritable variation in sinigrin concentration can be maintained by differential attack from specialists and generalists (Lankau 2007). However, despite intensive research on the supposedly coevolved interactions between secondary metabolite concentration and herbivore attack, direct ecological evidence for herbivore

responses to *structural* variation in secondary metabolites in wild plant populations is scarce. This may be because the fitness effects observed in laboratory assays are too subtle to be genuinely important in wild plant-herbivore communities, or because too few plants and plant populations are surveyed to give sufficient statistical power.

In *B. oleracea*, there exists significant variation in the type and concentration of aliphatic glucosinolates both within and between populations (Mithen et al. 1995b), but field surveys of four plant populations identified only two correlations between quantitative glucosinolate variation and herbivory: the moth *Selania leplastriana* (Tortricidae) is found in higher abundance on plants containing high concentrations of progoitrin (Moyes et al. 2000); and the weevil *Ceutorhynchus assimilis* (Curculionidae) is more prevalent on plants with high concentrations of gluconapin (Moyes and Raybould 2001).

While the results of these field surveys of *B. oleracea* (Moyes et al. 2000) are suggestive of links between glucosinolate concentrations and herbivore pressure, they provide little evidence that genetic polymorphisms in plant metabolites influence herbivory, for three reasons. First, although aliphatic glucosinolate concentration is partly under genetic control (Mauricio 1998; Rucker and Robbelen 1994), concentrations can also vary greatly with herbivore damage (Soler et al. 2005) and although total quantities of glucosinolates are relatively constant between populations, significant changes in aliphatic glucosinolate concentration occur throughout the year (Mithen et al. 1995b) even in controlled greenhouse conditions (Gols et al. 2007). Detection of top-down differential selection requires a study system with better-described heritability of anti-herbivore traits. Second, each plant population and its herbivore community cannot be considered a closed system: herbivore dispersal and oviposition choices occur both within and between plant populations. Therefore any lack of evidence of interactions between herbivores and secondary metabolites within a

plant population may simply reflect responses occurring on a larger ecological scale. Third, with only four populations of food plant the previous studies on responses of herbivores to glucosinolates (Moyes et al. 2000; Moyes and Raybould 2001) lacked sufficient statistical power to test differential herbivory hypotheses at the inter-population scale. What is required is evidence that herbivore species show differential responses to heritable plant phenotypes in wild populations.

An important but neglected aspect of this area of research, therefore, is how genetically controlled variation in plant defence chemistry structures the herbivore community in natural populations. In the field environment it is difficult to control for induction of secondary metabolites either by herbivores (Soler et al. 2005) or by variation in environmental conditions (such as nutrient availability (Zhao et al. 1994)). In this study we clarify the responses of specialist and generalist herbivores to *B. oleracea* plants that produce different aliphatic glucosinolates, based on variation caused by the presence or absence of functional alleles at two loci. Unlike quantitative variation in secondary metabolite concentrations, the ability of a plant to synthesise a compound is likely to be a form of variation that herbivores experience consistently in the field. Finding a link between natural herbivory rates and metabolic chemical variants with simple genetic mechanisms, would therefore provide a sound foundation for deeper study of the community genetics of plant-herbivore interactions.

We hypothesise that the scale at which herbivory pressures act may determine whether diversity in secondary metabolites, and structuring of the herbivore communities, is displayed within or between populations, or at both scales. Herbivores may distribute themselves among plants within populations according to their secondary metabolites, but randomly between populations. Herbivores may distribute themselves non-randomly with respect to population-scale metabolite profiles, but randomly within populations. In between these extremes, herbivores may distribute themselves non-

randomly between metabolite profiles of individual plants and of whole plant populations, leading to variation both within and between plant populations. The scale at which we detect patterns (if they exist) may depend critically on the dispersal ability of each herbivore species. If we are to investigate the potential for secondary metabolites to structure herbivore communities, which could in turn provide evidence for selection by herbivores as a mechanism maintaining variation in plant defences both within and between populations, we require surveys and experiments to be carried out at both scales. Recognising the importance of ecological scale, we replicate our surveys across 12 cabbage populations to improve statistical power.

Materials and Methods

FIELD SITES

Populations of wild cabbage are confined to maritime cliffs and the plant is morphologically and genetically distinct from cultivated varieties of *B. oleracea* which in Britain occur inland occasionally as feral populations (Preston et al. 2002). Twelve spatially distinct populations of *B. oleracea* across three counties in southwest England were used in this study: three populations in Cornwall: PC1 (50°10'N, 5°42'W), PC2 (50°12'N, 5°42'W), and PC3 (50°10'N, 5°41'W), three in Devon: WAT (50°50'N, 3°51'W), WH (50°46'N, 3°49'W) and KW (50°34'N, 3°56'W) and six in Dorset: OH (50°64'N, 1°92'W), DD (50°62'N, 2°27'W), AH (50°69'N, 2°05'W), WS1 (50°59'N, 2°03'W), WS2 (50°58'N, 2°04'W) and K2 (50°60'N, 2°13'W). At each population a minimum of 50 plants were selected and marked for glucosinolate analysis and herbivore survey.

SURVEYING PLANTS AND HERBIVORES

Plants selected were either at late rosette stage (close to flowering age), or were flowering for the first time in 2006. First flowering was determined by the absence of

the scars which would have resulted from previous flowering stems. A transect was laid at each site along which suitably-sized plants were selected at random and marked using individually-numbered copper tags. The transect coordinates and a 10 figure grid reference were recorded to enable the plant to be found for the following surveys. A leaf tissue sample was taken from each marked plant for glucosinolate profiling. Plants were surveyed twice for herbivore presence, in June and September 2006. Each marked plant was thoroughly examined for herbivores on the leaves and flowering stems and any evidence of past herbivory (such as exuviae of lepidopteran larvae, and mummified remains of parasitised aphids) was also recorded and included in the analysis of herbivore presence or absence (see sections on intra- and inter-population responses of herbivores to glucosinolate frequencies. Herbivores were identified according to Kirk (1992).

GENETICS OF GLUCOSINOLATES IN BRASSICACEAE

The Brassicaceae produce three classes of glucosinolates which differ in terms of their amino acid precursors: aliphatics, indoles and aromatics. Of these, aliphatic glucosinolates are under strong genetic control by five loci: *GSL-pro* and *GSL-elong* control the length of the side chain; *GSL-sulph*, *GSL-alk* and *GLS-oh* modify the side chain (Magrath et al. 1994; Mithen et al. 1995a; Parkin et al. 1994) (Fig. 2.1.). Gene products from some of the loci regulating side chain modification are not specific to the side chain length; interactions between the genes regulating side chain length and the genes regulating side chain modifications can occur, resulting in a mixture of glucosinolate structures within the plant (Mithen 2001).

In this study we investigated natural variation in the presence or absence of individual aliphatic glucosinolates, and not quantitative variation in glucosinolate concentrations. Indole glucosinolates are induced by herbivory (Bodnaryk 1992),

making it difficult to distinguish cause and effect in field surveys of herbivores and indole glucosinolates. Therefore, we focused our study on the presence/absence of aliphatic glucosinolates. Although quantitative differences in aliphatic glucosinolates will undoubtedly influence herbivore responses, increased concentrations may be induced by herbivory itself and will vary between plant tissues and between seasons (Mithen et al. 1995b; Soler et al. 2005). Therefore the presence or absence of individual compounds is likely to be what herbivores experience consistently in the field, is a known heritable component of the cabbage anti-herbivore defence system, and is a consistent variable when comparing responses of herbivores through time across populations.

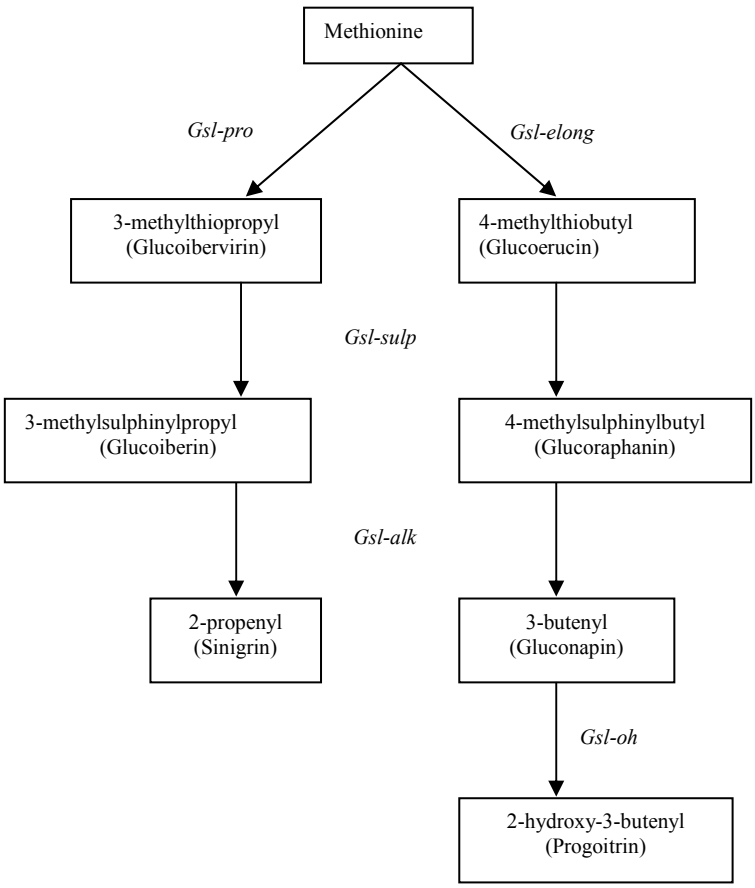

Figure 2.1. Diagram of aliphatic genetics (adapted from Mithen et al 1995a). Names of loci regulating side chain modification ate shown in italics

One fully developed intact leaf close to the centre of the rosette in non-flowering plants, or close to the base of the flowering stem in flowering plants, was removed from each marked plant. Leaves from this location on the plant were chosen because young leaves contain higher quantities of glucosinolates (Lambdon et al. 2003; Shelton 2005) and therefore would provide very clear signals of the presence or absence of individual glucosinolates. The leaf was boiled for 3 min immediately after removal from the plant using a Coleman (Bristol, UK) campstove in order to inhibit the action of the myrosinase enzyme, which would otherwise hydrolyse the glucosinolates (Prestera et al. 1996). The leaf samples were then returned to the lab, frozen and freeze-dried to constant weight. The glucosinolates were extracted and converted to desulphoglucosinolates according to published methods (Graser et al. 2000; van Dam et al. 2004). Freeze-dried plant material was ground to a coarse powder and 100mg placed into a ventilated 1.7ml microtube. The glucosinolates were extracted with boiling 70% methanol and desulphatased using sulphatase solution (prepared from Sigma-Aldrich type H-1 aryl sulphatase of Helix pomatia) on a DEAE Sephadex A-25 column (Sigma-Aldrich). The desulphoglucosinolates were eluted from the columns the following day. The samples were then freeze-dried and stored until required for HPLC.

The desulphoglucosinolates were separated using reverse-phase HPLC on a C18 Dionex column, with an acetonitrile - water gradient mobile phase (Spinks et al. 1984). The eluted desulphoglucosinolates were detected at 235nm using a Dionex PDA-100 Photodiode Array Detector, this wavelength was chosen because 3D spectra obtained from preliminary tests indicated this was where the highest peak in the spectra was observed and would therefore provide the clearest indication of the presence/absence of glucosinolates.

The glucosinolates were identified using sinigrin, gluconapin, progoitrin and glucoraphanin as external standards. The four aliphatic glucosinolate standards were converted to desulphoglucosinolates on Sephadex columns as described above for plant extracts. The retention times of peaks were also compared with published retention times (Kiddle et al. 2001; Mithen et al. 1995b; Spinks et al. 1984).

STATISTICAL ANALYSIS

All data analysis was carried out using mixed effects modelling in R version 2.5.1., with models simplified using likelihood ratio tests of significance. Standard model checks were used to verify normality and homogeneity of standardised residuals (Crawley 2007).

SPATIAL STRUCTURING OF GLUCOSINOLATE PROFILES AND HERBIVORY

The focus of our surveys was to investigate correlations between plant chemistry and herbivores occurring at the inter- and intra-population scales, but we have also included county and distances between plant populations in our analyses to absorb spatial autocorrelation of glucosinolate profiles and to provide information on the geographic structuring of plant and herbivore communities. The effect of population and county on the frequency of glucosinolates was analysed using a generalised linear model with binomial errors. We also used variance components from a generalised linear mixed effects model with binomial errors and population nested within county as random effects to study the partitioning of phenotypic variance between ecological scales. Further information on geographic structuring in this system was gained using Mantel tests (Mantel 1967) to determine whether glucosinolate profiles or herbivory were spatially autocorrelated. Mantel tests created similarity matrices, across populations, for geographic distance, glucosinolate frequencies and herbivore

presence/absence. Significant correlations between geographic similarity and similarity in herbivory or glucosinolates would suggest non-random spatial structuring of the variables measured in our surveys.

INTRA-POPULATION RESPONSES OF HERBIVORES TO GLUCOSINOLATES

To determine whether within-population variation in glucosinolate profiles influenced herbivore communities, the effect of the glucosinolates produced by each plant on the presence or absence of each herbivore species was analysed using generalised linear mixed effects models with a binomial error structure. The presence or absence of each glucosinolate, and survey timing, were included as fixed effects. We accounted for repeated measures (two surveys on each plant in each population) and spatial structuring by modelling plant identity as a random effect, nested within population, all nested within county.

INTER-POPULATION RESPONSES OF HERBIVORES TO GLUCOSINOLATE FREQUENCIES

Analysis of the responses of herbivores to glucosinolates at the population-scale was carried out using linear mixed effects models extended to include the effect of spatial autocorrelation in residuals according to Pinheiro and Bates (2000). The response variable for each model was the proportion of plants attacked by each herbivore, logit-transformed to normalise the residuals. Survey timing was included as a random effect, corrected for heteroscedasticity (difference in variance between surveys) using the VarPower command in R. We tested the influence of the proportion of plants containing each glucosinolate in the population, and survey timing, on the proportion of plants infested with each herbivore species. Recognising the existence of spatial autocorrelation in some of our response and explanatory variables, we used Akaike Information Criteria to choose a Gaussian autocorrelation function (from a set of

alternative functions), to describe the influence of geographic distance between populations on each model's residuals. This absorbing of spatial autocorrelation means that any significant responses of herbivores to population-scale glucosinolate frequencies, revealed by the mixed effects models, were real effects that were independent of any geographical arrangement of plant populations and herbivores.

MODELLING PROCEDURE AND MULTIPLE TESTING

The herbivores analysed in separate models were the specialists *Pieris rapae* (butterfly), *Pieris brassicae* (butterfly), *Aleyrodes proletella* (whitefly) and *Brevicoryne brassicae* (aphid), and the generalists *Mamestra brassicae* (moth), and snails (all snail species were analysed collectively). We tested the significance of fixed effects (glucosinolate identity, survey date) and their interactions using likelihood ratio tests and model simplification of maximum likelihood versions of the mixed effects models (Crawley 2007). In order to reduce type I errors a local false discovery rate (FDR) (Benjamini and Hochberg 1995) adjustment of *P*-values was applied to the intra- and inter-population scale tests using the fdrtool package for R (Strimmer 2008). False discovery rates were adjusted at the level of herbivore and ecological scale, rather than to the model simplification results within each herbivore analysis. Hence we present FDR-adjusted q-values for comparisons of the minimal adequate fixed effect models (the models that contained only significant fixed effects) to the null fixed effect models (containing no fixed effects), in Table 2.1.

Results

GEOGRAPHIC VARIATION IN GLUCOSINOLATES

The glucosinolates found were glucoiberin, sinigrin, glucoraphanin, gluconapin and progoitrin. All plants produced gluconapin and glucoraphanin, indicating that all

plants possess functional alleles at the *GSL-alk* and *GSL-sulph* loci. The variation in glucosinolate profiles was caused by the presence or absence of sinigrin (and its precursor glucoiberin) and progoitrin, arising from allelic variation at *GLS-pro* and *GSL-oh*. This variation results in each plant producing sinigrin, progoitrin, both, or neither (the 'neither' phenotype was found in only 3 plants out of a total of 598) (Fig. 2.2.).

The frequency of sinigrin and progoitrin presence varied significantly between populations (sinigrin, $\chi^2_{11}= 342$ $P < 0.01$; progoitrin, $\chi^2_{11} = 152$, $P < 0.01$). There were similarities in aliphatic glucosinolate profiles within counties. The Cornwall populations had only two phenotypes present in all three populations although the frequency of each phenotype varied. The Dorset populations had the most variable aliphatic glucosinolate profiles and there was no variation in the Devon populations (Fig. 2.2.). However the variation observed in glucosinolates cannot be fully explained by county: simplification of models comparing the frequency of phenotypes by replacing 'population' with 'county' resulted in a significant increase in residual deviance (sinigrin, $\chi^2_9= 21.1$, $P < 0.01$; progoitrin, $\chi^2_9 = 68.5$, $P < 0.01$). Variance components for the partitioning of variation between ecological scales are provided in Table 2.2. Mantel tests showed that the frequency of sinigrin production is negatively autocorrelated with distance between plant populations ('nearby' plant populations show similar sinigrin frequencies; $P = 0.01$), but that no such correlation exists for progoitrin frequency ($P = 0.28$).

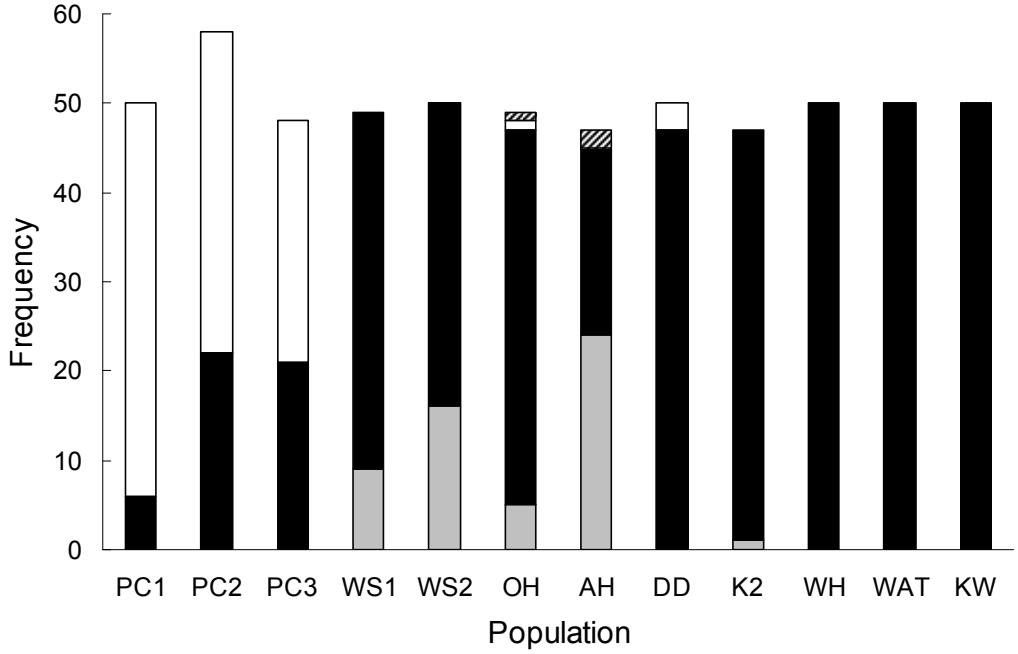

Figure 2.2. Frequencies of the four aliphatic glucosinolate phenotypes in each population. Black bars = sinigrin and progoitrin present in the plant, white bars = progoitrin only, grey bars = sinigrin only and hatched bars = both absent. Cornwall populations = PC1, PC2 and PC3. Dorset populations = WS1, WS2, OH, AH, DD and K2. Devon populations = WH, WAT and KW.

Table 2.1. Significance of all fixed factors tested in the generalised linear mixed effects models for each herbivore. The fixed effects column and its corresponding *P*-value shows the significance of each main effect or interaction tested (main effects could only be tested if they were not involved in any significant interactions). The number of plants infested by each herbivore (n), the minimal adequate model (MAM; the simplest model that contained only significant fixed effects) and its overall significance (tested against the null model) as well as its false discovery rate-adjusted q-value are shown. The q-values control the scale-wide false discovery rate to have $\alpha = 0.05$ and so can be interpreted in the same way as conventional *P*-values.

Scale	Herbivore	n	MAM	Fixed effects	*P*-value	MAM vs. null (*P*-value)	MAM vs. null (*q*-value)
Intra-population	*B. brassicae*	150	sinigrin*survey	sinigrin:survey sinigrin: progoitrin progoitrin:survey progoitrin	0.03 0.55 0.20 0.24	<0.001	<0.001
	P. brassicae	57	sinigrin*survey	sinigrin:survey sinigrin: progoitrin progoitrin:survey progoitrin	<0.01 0.80 0.55 0.62	<0.001	<0.001
	Snail	361	sinigrin*survey	sinigrin:survey sinigrin: progoitrin progoitrin:survey progoitrin	0.02 0.27 0.14 0.34	<0.001	<0.001
	P. rapae	222	NULL	sinigrin:survey sinigrin: progoitrin progoitrin:survey progoitrin sinigrin	0.28 0.26 0.10 0.16 0.86	0.431	0.517
	A. proletella	330	NULL	sinigrin:survey sinigrin: progoitrin progoitrin:survey progoitrin sinigrin	0.19 0.41 0.83 0.16 0.42	0.313	0.417
	M. brassicae	19	NULL	sinigrin: progoitrin progoitrin sinigrin	0.79 0.37 0.35	0.847	0.847

Table 2.1. continued:

Scale	Herbivore	n	MAM	Fixed effects	*P*-value	MAM vs. null (*P*-value)	MAM vs. null (*q*-value)
Inter-population	*B. brassicae*	150	sinigrin*survey + progoitrin*survey	sinigrin:survey sinigrin: progoitrin progoitrin:survey	0.03 0.94 0.04	0.020	0.035
	P. brassicae	57	sinigrin*progoitrin	sinigrin:survey sinigrin: progoitrin progoitrin:survey survey	0.20 0.04 0.79 0.51	0.048	0.071
	Snail	361	sinigrin+survey	sinigrin:survey sinigrin: progoitrin progoitrin:survey progoitrin sinigrin survey	0.37 0.38 0.94 0.74 0.007 0.02	0.003	0.005
	P. rapae	222	NULL	sinigrin:survey sinigrin: progoitrin progoitrin:survey progoitrin sinigrin survey	0.96 0.77 0.57 0.61 0.10 0.31	0.314	0.377
	A. proletella	330	sinigrin*survey	sinigrin:survey sinigrin: progoitrin progoitrin:survey progoitrin	0.002 0.99 0.21 0.28	<0.001	0.002
	M. brassicae	19	survey	sinigrin: progoitrin progoitrin :survey sinigrin :survey sinigrin progoitrin survey	0.60 0.33 0.54 0.21 0.20 0.002	0.002	0.005

Mantel tests of spatial autocorrelation in herbivore frequencies demonstrated that *A. proletella* infestation was negatively autocorrelated with distance between plant populations ('nearby' plant populations show similar whitefly frequencies; $P = 0.02$), however we found no significant spatial autocorrelation in herbivore frequency for *B. brassicae* ($P = 0.87$), *P. brassicae* ($P = 0.47$), snails ($P = 0.71$), *P. rapae* ($P = 0.07$) or *M. brassicae* ($P = 0.87$). This is also supported by the variance components of the random effects in the intra-population scale analysis which indicate that county accounts for very little of the variation in herbivore presence for all herbivore species with the exception of *A. proletella* (Table 2.2). Conversely, population appears to be a very important scale in the structuring of herbivore frequencies (Table 2.2).

Table 2.2. Variance components for each ecological scale shown for all herbivore species and the glucosinolates sinigrin and progoitrin. For *M. brassicae* only the variance componants for population and county are presented as this species was only observed in the early season survey and therefore we limited the analysis of the effect of glucosinolates in *M brassicae* to survey 1. There was no within plant variation in glucosinolates as leaf collection and HPLC analysis was carried out once for each plant

	% Variance explained by:		
Herbivore	Plant ID	Population	County
B. brassicae	48.6	50.5	0.9
P. brassicae	99.9	<0.1	<0.1
Snail	15.8	69.2	15.0
P. rapae	1.1	77.2	21.7
A. proletella	12.9	22.0	65.1
M. brassicae	NA	99.0	1.0
Glucosinolate			
Sinigrin	NA	7.6	92.4
Progoitrin	NA	6.8	93.2

All *P*-values for the analysis of herbivore responses to glucosinolates at the intra- and inter-populations scales, together with the false discovery rate adjusted *q*-values, are presented in Table 2.1. Within populations, herbivore species showing significant responses to sinigrin were the aphid *B. brassicae,* the butterfly *P. brassicae* and snails. The effect of this glucosinolate varied depending on time of year: *B. brassicae* was found in higher abundance on plants lacking sinigrin in June but this effect was reduced in September (Table 2.1; Fig. 2.3a). *Pieris brassicae* showed a positive association with plants producing sinigrin in June, but were more likely to be found on plants lacking sinigrin in September (Table 2.1; Figure 2.3b). Snails also showed a significant response to the interaction between sinigrin and survey timing: higher densities of snails were observed on plants producing sinigrin in June but this response was less pronounced in September (Table 2.1; Figure 2.3c). None of these herbivores showed any evidence of a response to the interaction between sinigrin and progoitrin, or responded to the presence or absence of progoitrin. The herbivores *Mamestra brassicae, A. proletella* and *P .rapae* did not demonstrate significant responses to either glucosinolate at the intra-population scale (Table 2.1).

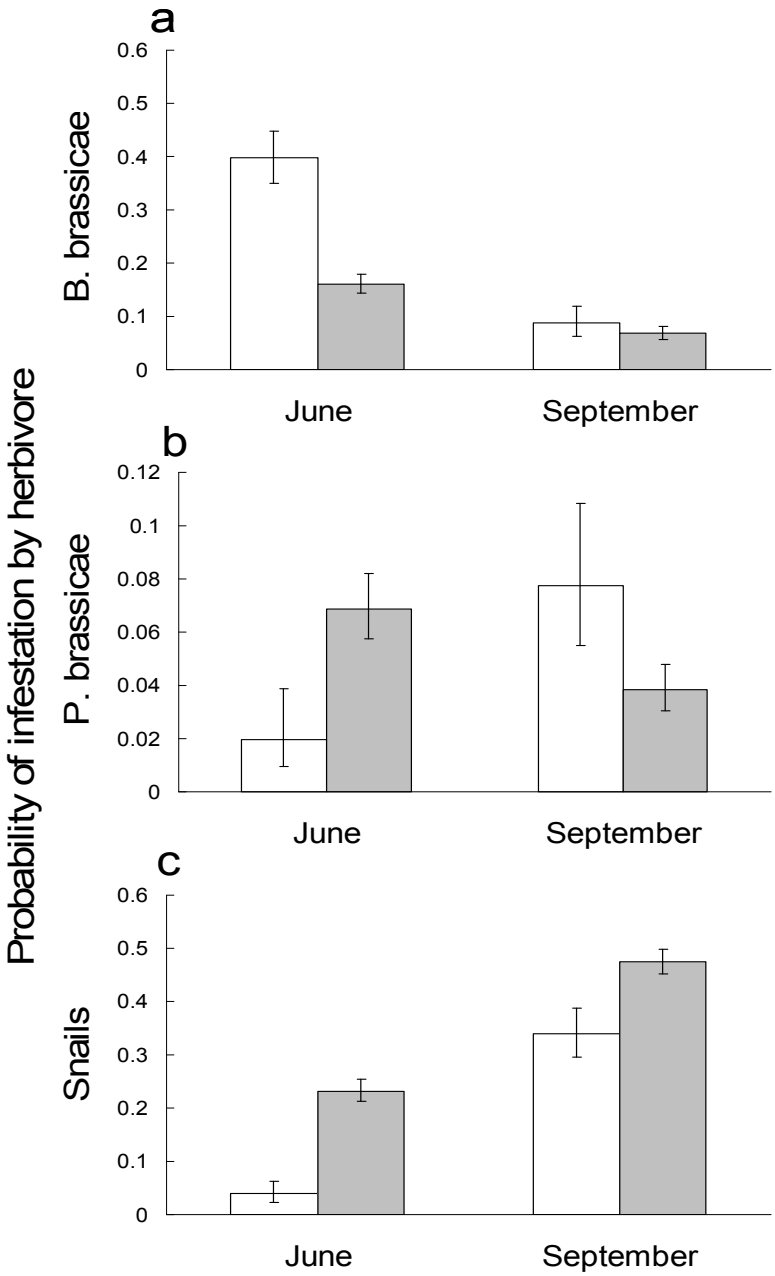

Figure 2.3 Responses of herbivore species to the presence or absence of sinigrin in plants within populations. Mean probability of a plant lacking (white bars) or producing (grey bars) sinigrin being infested with *B. brassicae* (a), *P. brassicae* (b) and snails (c)**.** Means and standard errors calculated from generalised linear models of herbivore response to individual glucosinolates, ignoring correlated response to other glucosinolates and the random effects of population and plant ID. Hence the graphs show the raw, main effects of sinigrin presence.

At the inter-population scale, having accounted for spatial autocorrelation in residuals, the proportion of plants infested with *B. brassicae, P. brassicae, A. proletella* and snails showed a significant response to the proportion of plants producing sinigrin (Table 2.1). There was no significant effect of plant chemistry on *P. rapae* or *M. brassicae* presence at this scale.

The infestation rate of *B. brassicae* was higher in populations with a low proportion of plants producing sinigrin in June but this response was lost in September (Fig. 2.4a). *B. brassicae* also showed a response to progoitrin at the inter-population scale, exhibiting higher infestation rates in populations with high proportions of plants producing progoitrin in June: however, the effect of progoitrin only became significant in multiple regression models that included the sinigrin effects (Table 2.1; Fig. 2.4b). Having accounted for the negative correlation between aphid infestation and the proportion of plants producing sinigrin, the extra effect of progoitrin-producing plants on aphid infestation was also negative. As there was no evidence of an interaction between sinigrin and progoitrin, this rather confusing result, which contradicts the positive correlation suggested by Fig. 2.4b, was due to correlations across populations between the number of plants producing sinigrin and those producing progoitrin. It is impossible to determine from our survey results whether the apparent positive effect of progoitrin on aphid infestation (Fig. 2.4b) was due to progoitrin *per se*, or an artefact of the effect of correlated sinigrin-production by plants at the population level.

A. proletella showed no response to sinigrin and progoitrin at the intra-population scale but a significant response to the interaction between sinigrin and survey time was detected at the inter-population scale. Infestation by *A. proletella* decreased with higher proportions of plants producing sinigrin but the rate of decrease

was slightly lower in the early season survey (Table 2.1, Fig, 2.4c). Progoitrin did not influence *A. proletella* infestation.

Snails showed a positive association with sinigrin at the inter-population scale but there was no interaction between sinigrin and survey time (Table 2.1; Fig. 2.4e). The proportion of plants producing progoitrin (Fig. 2.4f) and the interaction between sinigrin and progoitrin did not influence the infestation rate of snails at the population scale.

P. brassicae presence was influenced by the interaction between sinigrin and progoitrin (Table 2.1). Sinigrin and progoitrin both had a positive effect on *P. brassicae* infestation and showed no interaction with survey timing, but the interaction between sinigrin and progoitrin was negative. This means that as the proportion of plants in a population producing both glucosinolates increases, the positive main effects of sinigrin and progoitrin on *P. brassicae* infestation rate showed diminishing returns. However, this result did not remain significant after FDR correction (Table 2.1), so we do not graph or discuss these results further.

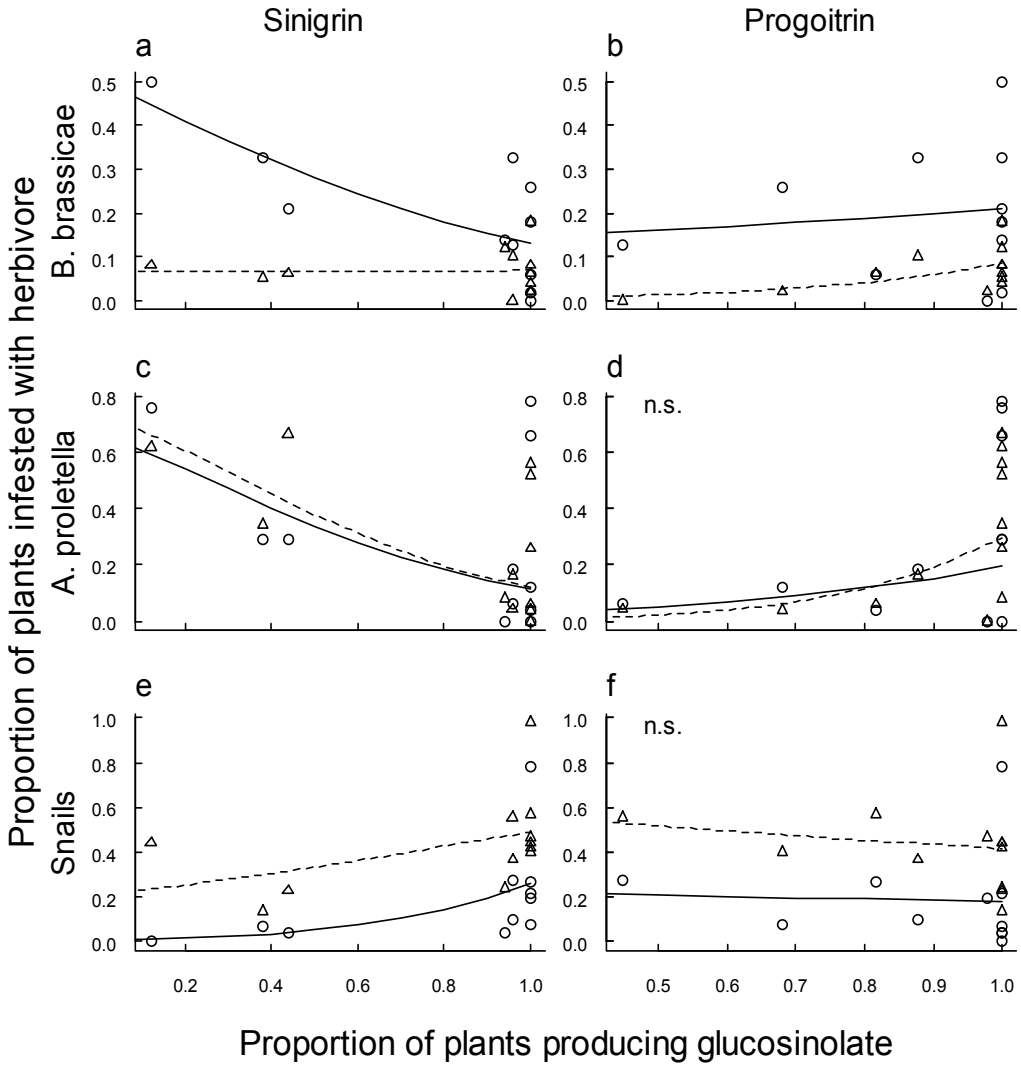

Figure 2.4. Herbivores showing significant responses to glucosinolates at the inter-population scale. Results for June (circles and solid line) and September (triangles and dotted line) surveys. Fitted lines are based on generalised linear models of herbivore responses (presence or absence) to individual glucosinolates, ignoring correlated influence of other glucosinolates and the random effect of population. Hence regression lines show the raw, main effects of glucosinolate frequencies, while significance indicators refer to mixed effects models. This allows fitted lines to fit the data visually, and avoids shrinkage of best linear unbiased predictors in maximum likelihood mixed effect models (Crawley, 2007). Graphs of herbivore responses to both glucosinolates are shown but non-significnat trends are marked with "n.s.".

Discussion

The effect of glucosinolate structural variation on six herbivore species was investigated across two ecological scales. Within populations, *Brevicoryne brassicae* exhibited a negative response to sinigrin which varied with time of year. *Pieris brassicae* and snails exhibited a positive response to sinigrin in June, but the direction of the responses of these herbivores changed with time of year. At the scale of whole populations, *B. brassicae* retained a negative correlation with the frequency of plants producing sinigrin, and snails retained a positive response to sinigrin. The positive response of *P. brassicae* to sinigrin was not detected at the inter-population scale. However, two additional correlations were revealed at the inter-population scale only: *B. brassicae* frequency showed a significant positive response to the frequency of plants producing progoitrin, and *A. proletella* frequency correlated negatively with the frequency of sinigrin-producing plants.

The glucosinolates showed significant spatial structuring and there were similarities in plant chemistry within counties which was controlled for during the analysis of the responses of herbivores to plant chemistry. The observed spatial autocorrelation may be due to gene flow between populations which has implications for the introduction and maintenance of genetic diversity (Slatkin 1985). Gene flow between *B. oleracea* populations is probable: pollinator-mediated gene flow is likely as distance between several of the populations is less than the potential range of many bee species (around 10km) (Pasquet et al. 2008; Visscher and Seeley 1982) and molecular marker analysis has demonstrated significant gene flow between populations of *B. oleracea* in Dorset (Raybould et al. 1999). As all the populations are on or near the south west coast path, human-mediated dispersal could have a significant impact on gene flow: *B. oleracea* seeds can be carried over 5km on walker's boots (Wichmann et al. 2009).

There are several ecological mechanisms that may explain differences in the responses of different herbivore species to different glucosinolates. *Brassica* specialists have a wide variety of resistance mechanisms to the glucosinolate defence system. For example, the moth *Plutella xylostella* produces a sulphatase enzyme that prevents the glucosinolate from being hydrolysed upon damage to the plant (Ratzka et al. 2002; Wittstock et al. 2004), whereas *P. rapae* possesses an enzyme that redirects glucosinolate hydrolysis to the production of nitriles which the herbivore can then excrete (Wittstock et al. 2004). Hence, variation in resistance mechanisms may cause herbivores to have different susceptibilities to the various glucosinolates. Feeding mode may also result in different herbivore responses; the distribution of glucosinolates around the plant is highly variable, such that total concentration decreases with age in leaves (Lambdon et al. 2003; Shelton 2005), even varying on small spatial scales within leaves (Shelton 2005) and cells with high concentrations of glucosinolates have been identified around the phloem of *Arabidopsis* flowering stems (Koroleva et al. 2000). Therefore herbivores feeding on different plant parts may be subjected to different glucosinolate concentrations. The differential responses of herbivores may be caused by signals at the oviposition level in some species: *Pieris* species have been shown to vary in the strength of their oviposition response to structural variation in glucosinolates (Huang and Renwick 1994) and infestation by *A. proletella* may be determined at the oviposition stage as the nymphs usually settle next to the eggs from which they hatch and are immobile after the first moult (Resh and Carde 2003). Alternatively, a higher trophic level may be involved: parasitoids show variation in attraction to structural (Raybould and Moyes 2001) and quantitative (Bradburne and Mithen 2000) differences in glucosinolates. If plant chemistry affects parasitoid host-location efficiency (De Moraes et al. 1998) the variation in herbivore preference may be driven by the need to avoid natural enemies (the enemy free space hypothesis; Jefferies & Lawton (1984)).

SEASONAL VARIATION IN HERBIVORE RESPONSES

Within herbivore species, time of year also affected the responses of the herbivores, a phenomenon that has been previously observed in arthropod communities on Cottonwood stands (Wimp et al. 2007). Temporal variation in responses within a species may be caused by quantitative changes in glucosinolates within the plant (Gols et al. 2007; Mithen et al. 1995b) or could be mediated by natural enemies and parasitoids of the herbivore: the differences in preference at the end of the season may simply reflect the after-effects of predation or parasitism by natural enemies which used secondary metabolites as host location cues. *B. oleracea* is perennial so individual plants will experience inter-annual variation in the herbivore community. It is also likely that the impact of herbivores on plant fitness varies during the year: plants may be more vulnerable to herbivores during the flowering season or during periods of rapid growth. Thus total lifetime reproductive success of this perennial plant will depend on changes in the herbivore community through time.

IMPORTANCE OF ECOLOGICAL SCALE

Several laboratory studies have detected signals of herbivore responses to structural variation in plant defence compounds (Berenbaum and Zangerl 1998; Castellanos and EspinosaGarcia 1997; Huang and Renwick 1994; Huang and Renwick 1993; Linhart and Thompson 1995), however studies of natural populations often report no significant effect (Latta and Linhart 1997; Moyes et al. 2000), or have only detected responses at the population scale (Moyes and Raybould 2001). We believe this is often because too few plants, or too few populations, have been surveyed in previous studies (Latta and Linhart 1997; Moyes et al. 2000; Moyes and Raybould 2001). The results presented here, which gain increased power through improved replication, contradict previous results by finding differential responses of four herbivore species to sinigrin either

within or between populations or at both scales. Natural herbivore-plant communities are not closed systems and our analysis of the variance components for the three spatial scales (plant, population and county) demonstrated the importance of investigating herbivore responses at the population scale.

B. brassicae and snails showed a consistent negative correlation with sinigrin at both scales, suggesting that their preference for plant metabolic phenotypes may simply scale up to population level patterns, or vice versa. The within-population preference of *P. brassicae* for plants producing sinigrin did not scale up to a preference for plant populations containing higher frequencies of sinigrin-producing plants. We speculate that this lack of scaling may be due to the scale at which this butterfly selects foodplants: attraction to patches of plants may be mediated by visual or coarse biochemical attraction, while distribution of oviposition decisions within populations may depend on specific plant chemistry. Interestingly, the whitefly *A. proletella* showed no preference for plant metabolic phenotypes within populations, but frequencies of this herbivore were lower in plant populations with low frequencies of sinigrin-producers, over and above the significant spatial structuring of this herbivore's geographic distribution. It is intriguing that the two small herbivore species that have passive dispersal mechanisms (aphids and whitefly tend to be dispersed across large distances by wind (Compton 2002)) were those that displayed population-scale patterns of response to glucosinolate profiles, along with snails that would not be expected to make herbivory decisions at the population scale due to limited dispersal options.

The results for progoitrin were much weaker with only *B. brassicae* exhibiting a response at the inter-population scale; despite this weaker correlation with herbivores, variation in progoitrin is still maintained at both ecological scales. It is possible that our survey lacked sufficient power to detect species-specific responses to progoitrin within populations. An alternative explanation of within-population variation in progoitrin

production is that it may be maintained, despite its weak association with herbivory, by correlated production of this glucosinolate alongside sinigrin. It is also important to note that the previous field studies tend to focus on quantitative rather than qualitative variation in plant secondary compounds and this may also be a reason for the disparity between the results presented here and previous work (Latta and Linhart 1997; Moyes et al. 2000), although Moyes and Raybould (2001) only detected a response to glucosinolate concentration by seed weevils at the population scale and found no effect of gluconapin concentration on weevil oviposition at the plant level.

IMPLICATIONS FOR THE MAINTENANCE OF SECONDARY METABOLITE DIVERSITY

Three rival hypotheses for the maintenance of secondary metabolite variation can be considered. First, selection for or against glucosinolates may be weak. This weak selection hypothesis states that whatever the evolutionary history of the plant's metabolism, the current fitness costs of producing different defence chemicals, and the selection pressures imposed by herbivores, is negligible. This hypothesis has strong links with the neutral theories of evolution (Kimura 1991) and biodiversity (Hubbell 2001). In this scenario, metabolic diversity is simply drifting through ecological time and is maintained via mutation and pollen or seed transfer between plant populations. Alternatively, if plant fitness is influenced by glucosinolates then 'fluctuating' or 'differential' selection may maintain glucosinolate diversity (Bossart and Scriber 1995; Gillespie and Turelli 1989; Lankau 2007). Such selection pressures may be mediated by the abiotic environment (hypothesis two: bottom-up differential selection) or by annual, seasonal or scale-dependent variation in herbivory (hypothesis three: top-down differential selection). The results presented here show a correlation between structural variation in glucosinolates and herbivore species in the field and lend support to the idea that intra- and inter-population variation in defence chemical composition of *B.*

oleracea may be maintained by top-down differential selection. The survey results indicate that this area of research merits more detailed investigations into the effects of plant chemistry on herbivore fitness and also the effects of herbivore attack on plant fitness.

GENERALIST VS. SPECIALIST HERBIVORE SPECIES

The herbivores in this study can be grouped into suites of species, based on similar responses to the glucosinolates: *P. brassicae* and snails are more likely to be found on plants producing sinigrin, while *B. brassicae* prefers plants lacking sinigrin, with *P. rapae* showing a similar (but non-significant) trend. *P. brassicae, B. brassicae,* and *P. rapae* are specialists on plants in the *Brassicaceae*, whereas snails are generalists. There are a number of conflicting studies that support (Gols et al. 2008a; Lankau 2007) and reject (Agrawal 2000; Poelman et al. 2008) the hypothesis that the response of herbivores to glucosinolate concentration can be categorised by whether the species are generalist or specialist. This conflict remains true for the influence of structural differences in glucosinolates: *M. brassicae* (a generalist lepidopteran) larvae developing on *Barbarea vulgaris* plants were affected by structural variation in glucosinolates, however, the specialist lepidopteran *P. rapae* showed no response to glucosinolate phenotype (van Leur et al. 2008). Our survey found that the suites of herbivores showing similar responses to glucosinolates do not match with their classification as generalists and specialists (based on foodplant species diversity).

Coevolutionary arms races seem to have driven evolution of specialism in many herbivores (Ehrlich and Raven 1964), whereby metabolites that deter generalists have become attractants or feeding stimulants for specialists (Huang and Renwick 1994; Nielsen et al. 1989). This apparent dichotomy in the fitness impacts of metabolites has led to a refinement of the top-down differential selection hypothesis: antiherbivore

metabolite diversity may be maintained by a balance between the selection pressures imposed by specialist vs. generalist herbivore species in natural populations (Lankau 2007). We do not find that herbivores showing similar responses to glucosinolates can be grouped into generalists and specialists. It is possible that the generalist vs. specialist hypothesis holds for some aspects of secondary metabolite variation: indole glucosinolates may play a greater role in defence against specialists whereas all glucosinolates have a deterrent effect on generalists (Gols et al. 2008b). Alternatively, differences in the responses of generalist and specialist herbivores may become clear when considering quantitative variation in individual secondary compounds (Lankau 2007). Selection on plant defence compounds could be mediated by groups of herbivores united by similar responses to the host chemicals (Maddox and Root 1990), but the members of the different groups are not necessarily defined by the host plant range of the herbivore. Instead, we propose that differential selection may be mediated by each specific herbivore's preference for or avoidance of heritable plant defences, and intra- and inter-annual variation in the intensity of herbivory by each species. We emphasise that community structuring of herbivores depends on distribution processes operating both within and between plant populations.

We have provided evidence indicating that the structure of herbivore communities can be influenced by secondary metabolite diversity in wild plant populations, and that different structuring can happen at different times of year and at different ecological scales. These results could be exploited to help minimise herbivory in cultivated *Brassica* crops. However our main motivation is to understand why so much secondary metabolite variation exists in plant populations. The signals of herbivore responses detected in this study are only the first step in elucidating the mechanisms maintaining herbivore community structure and secondary metabolite diversity. Surveys of herbivore responses to secondary metabolites, such as those

described here, may fail to capture cause and effect in this system, therefore we recommend the development of experimental manipulations of glucosinolate frequencies in controlled field experiments. Finally, conclusive evidence of herbivore-mediated differential selection will require a demonstration that differential attack by herbivores in the field has differential impacts on plant fitness.

Acknowledgements

The authors thank J. Blount, A. Barlow and N. van Dam for providing advice on HLPC and techniques for purifying desulphoglucosinolates, Matthias Wichmann for advice about the Dorset sites and Isobel Giblin for fieldwork assistance at the Devon sites. P. Behrens, T. Jones and Torbay Coast and Country Side Trust allowed access to field sites. Funding for this project was provided by the European Social Fund. All experiments comply with current UK laws.

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Chapter 3

Inter-annual responses of herbivores to glucosinolate polymorphisms in wild cabbage (*Brassica oleracea*)

Abstract

1. Natural populations of wild cabbage (*Brassica oleracea*) show significant qualitative and quantitative diversity in aliphatic glucosinolates, a class of secondary metabolites involved in defence against herbivore attack. A previous one year survey in this system demonstrated that herbivore species show differential responses to the variation in plant chemistry across two ecological scales: within and between populations. The results demonstrated that heritable variation in plant antiherbivore compounds can structure the herbivore community.

2. The variation in the preferences of different herbivore species for the presence or absence of plant defence chemicals provides some evidence that top-down differential selection may play a role in the maintenance of glucosinolate diversity. However, to investigate how the structuring of herbivore communities may promote the maintenance of plant chemical diversity it is necessary to investigate correlations between herbivores and glucosinolates over a longer time period.

3. An extended survey in natural wild cabbage populations was carried out in order to investigate the responses of herbivore species within and between populations over three years.

4. The responses of *B. brassicae* and snails to the presence or absence of sinigrin were highly consistent. Several other herbivore species also showed significant, but more variable responses to sinigrin. *B. brassicae, A. proletella, P. brassicae* and *P. rapae* also showed significant and temporally variable response to progoitrin. The results strengthen the conclusions from the 2006 survey that variation in plant secondary metabolites can structure the local herbivore community and uphold the suggestion that

herbivore-mediated differential selection is a strong contender as a mechanism maintaining the observed diversity of glucosinolates in wild cabbage populations.

Introduction

A wide number of plant taxa show substantial intra- and inter-population variation in secondary metabolites involved in herbivore deterrence (Berenbaum et al. 1986; Mithen et al. 1995; Moyes et al. 2000; Richards and Fletcher 2002; Harvey et al. 2005; van Leur et al. 2006). This diversity in secondary metabolites challenges the evolutionary prediction that the optimum compound or chemical profile for defence against herbivore attack should spread to fixation in a population.

There are three mechanisms that could be operating to maintain the observed variation in secondary metabolites in natural populations. First, following the neutral theories of evolution (Kimura 1991) and biodiversity (Hubbell 2001), selection on secondary metabolite variation may be negligible and so diversity is maintained by genetic drift and gene flow between populations. The two alternative hypotheses assume that secondary metabolites influence plant fitness, and involve fluctuating or differential selection pressures (Ehrlich and Raven 1964). The selection pressures acting on secondary metabolites may be mediated by the abiotic environment (hypothesis two), resulting in bottom-up differential selection. Alternatively, as the diversity in secondary metabolites probably evolved through an evolutionary arms race between herbivores and plants (Fahey et al. 2001), top-down differential selection mediated by herbivores may also be involved in the maintenance of this diversity. A key requirement for top-down differential selection is that herbivore species present in the community should show differential responses to the plant chemical variants.

Glucosinolates compose a diverse class of secondary metabolites found in the Brassicaceae plant family. *Brassica* plants produce three classes of glucosinolates which

differ in the amino acid used in their biosynthesis: aliphatics, aromatics and indoles (Mithen et al. 1995; Moyes et al. 2000). The genetic control of aliphatic glucosinolate biosynthesis is well characterised and the ability of a plant to synthesise individual aliphatics is determined by allelic variation at 5 loci (see Chapters 1 and 2 for a full description). Natural populations of wild cabbage (*Brassica oleracea* L. var. *oleracea*, subsequently referred to as *B. oleracea*) show significant inter- and intra-population variation in aliphatic glucosinolates (Crawley 2007). Previous work on natural wild cabbage populations indicates that the local herbivore community may respond variably to differences in plant chemistry (Chapter 2), which provides some support for the hypothesis that intra- and inter-population variation in defence chemical composition of *B. oleracea* may be maintained by top-down differential selection. In Chapter 2 we proposed that differential selection may be mediated by each specific herbivore's preference for or avoidance of heritable plant defences, combined with intra- and inter-annual variation in the intensity of herbivory by each species. In order to test these two assumptions and to provide further support for this hypothesis, it is necessary to study the responses of herbivores to variation in glucosinolates over more than one year.

We can expect one of several possible outcomes: first, the responses of herbivores to glucosinolate variation that was observed in the previous study (Chapter 2) may be consistent and the relative abundance of herbivore species fluctuates over time. Second, the preferences of herbivores for different glucosinolates may vary but the abundance of herbivores is consistent between years. Third, the preferences of herbivores and the relative abundance of different species both show changes over time. Finally, the responses of herbivores and their abundance may be consistent. In this case the diversity of plant chemical phenotypes could be held at equilibrium by the differential responses to glucosinolate shown by species present in the herbivore community.

Materials and Methods

The methods used for herbivore surveys and determining the aliphatic glucosinolate profiles of wild cabbage plants are as described in Chapter 2. In 2007 and 2008 additional plants were selected and marked, using the same selection criteria as in 2006, to replace plants that had died during the previous year in order to maintain approximately 40-50 survey plants in each population. The glucosinolate profiles of the new plants showed one of the 4 phenotypes described in Chapter 2: all variation in aliphatic glucosinolate profiles was caused by the presence or absence of sinigrin (2-propenyl) and progoitrin (2-hydroxy-3-butenyl).

STATISTICAL ANALYSIS

Analysis of herbivore responses to glucosinolates was carried out at the intra- and inter-population scales as described in Chapter 2. All data analysis was carried out using mixed effects modelling in R version 2.7.0, with models simplified using likelihood ratio tests of significance. Standard model checks were used to verify normality and homogeneity of standardised residuals (Pinheiro and Bates 2000).

INTRA-POPULATION RESPONSES OF HERBIVORES TO GLUCOSINOLATES

To determine whether within-population variation in glucosinolate profiles influenced herbivore communities, the effect of the glucosinolates produced by each plant on the presence or absence of each herbivore species was analysed using generalised linear mixed effects models with a binomial error structure. The presence or absence of each glucosinolate, survey timing, and year of survey were included as fixed effects. We accounted for repeated measures (two surveys on each plant in each population over three years) and spatial structuring by modelling plant identity as a random effect, nested within population, all nested within county.

Analysis of the responses of herbivores to glucosinolates at the population-scale was carried out using linear mixed effects models, extended to include the effect of spatial autocorrelation in residuals (Crawley 2007). The response variable for each model was the proportion of plants attacked by each herbivore, logit-transformed to normalise the residuals. Survey timing nested within year were included as random effects, corrected for heteroscedasticity (difference in variance between surveys) using the VarPower command in R. We tested the influence of the proportion of plants containing each glucosinolate in the population, survey timing, and year on the proportion of plants infested with each herbivore species. Recognising the existence of spatial autocorrelation in some of our response and explanatory variables, we used Akaike Information Criteria to choose a Gaussian autocorrelation function (from a set of alternative functions), to describe the influence of geographic distance between populations on each model's residuals. This absorbing of spatial autocorrelation means that any significant responses of herbivores to population-scale glucosinolate frequencies, revealed by the mixed effects models, were real effects that were independent of any geographical arrangement of plant populations and herbivores.

MODELLING PROCEDURE AND MULTIPLE TESTING

The herbivores analysed in separate models were the specialists *Pieris rapae* (butterfly), *Pieris brassicae* (butterfly), *Aleyrodes proletella* (whitefly), *Brevicoryne brassicae* (aphid), and generalist snails (all snail species were analysed collectively). We tested significance of fixed effects (glucosinolate identity, survey date and year) and their interactions using likelihood ratio tests and model simplification of maximum likelihood versions of the mixed effects models (Crawley 2007). In order to reduce type I errors a local false discovery rate (FDR)(Benjamini and Hochberg 1995), adjustment

of *P*-values was applied to the intra- and inter-population scale tests using the fdrtool package for R (Strimmer 2008). False discovery rates were adjusted at the level of herbivore and ecological scale, rather than to the model simplification results within each herbivore analysis. Hence we present FDR-adjusted q-values for comparisons of the minimal adequate fixed effect models (the models that contained only significant fixed effects) to the null fixed effect models (containing no fixed effects), in Table 3.1.

Results

WITHIN POPULATIONS

All *P*-values for the analysis of herbivore responses to glucosinolates at the intra- and inter-populations scales, together with the false discovery rate adjusted *q*-values, are presented in Table 3.1. At the intra-population scale *B. brassicae, A. proletella* and *P. rapae* responded to the presence of sinigrin and progoitrin, while snails showed a response to sinigrin only. Plants producing sinigrin were less likely to be infested with *B. brassicae* and the extent of this effect varied between years. Conversely, this herbivore was observed more frequently on plants producing progoitrin (Table 3.1, Figs 3.1a & b). Survey also had a significant impact on *B. brassicae* infestation with fewer plants infested in the late season survey: there was no interaction between survey timing and either glucosinolate (Table 3.1). *Pieris rapae* showed significant interaction between sinigrin presence and year (Table 3.1). Figure 3.1c shows a negative response to sinigrin in 2006, but in 2007 and 2008 (when the abundance of *P.* rapae was low) there appears to be no effect of sinigrin. In addition the effect of progoitrin on *P. rapae* was also significant and showed even greater inter-annual variation (Table 3.1): Figure 3.1d shows a positive, negative and non-significant response of *P. rapae* to progoitrin presence over the three survey years. Survey time also significantly affected *P. rapae* presence: a greater number of plants were observed

to be infested with this herbivore in the late season surveys (Table 3.1). The presence of *A. proletella* was also correlated with both glucosinolates (Table 3.1): plants producing sinigrin and plants lacking progoitrin were less likely to be infested with whitefly. The effect of both glucosinolates on *A. proletella* varied between years (Table 3.1 Figs 3.1.e & f). Snail presence or absence was significantly affected by sinigrin but this effect depended on survey time (Table 3.1): snails showed a positive response to sinigin in the June surveys but this effect was reduced in the late season surveys (Fig. 3.2). Progoitrin had no significant effect on snail presence and year was significant as a main effect but showed no interactions with sinigrin or progoitrin. *P. brassicae* presence was not linked to glucosinolate profile: the presence of this herbivore varied with year and survey timing only (Table 3.1).

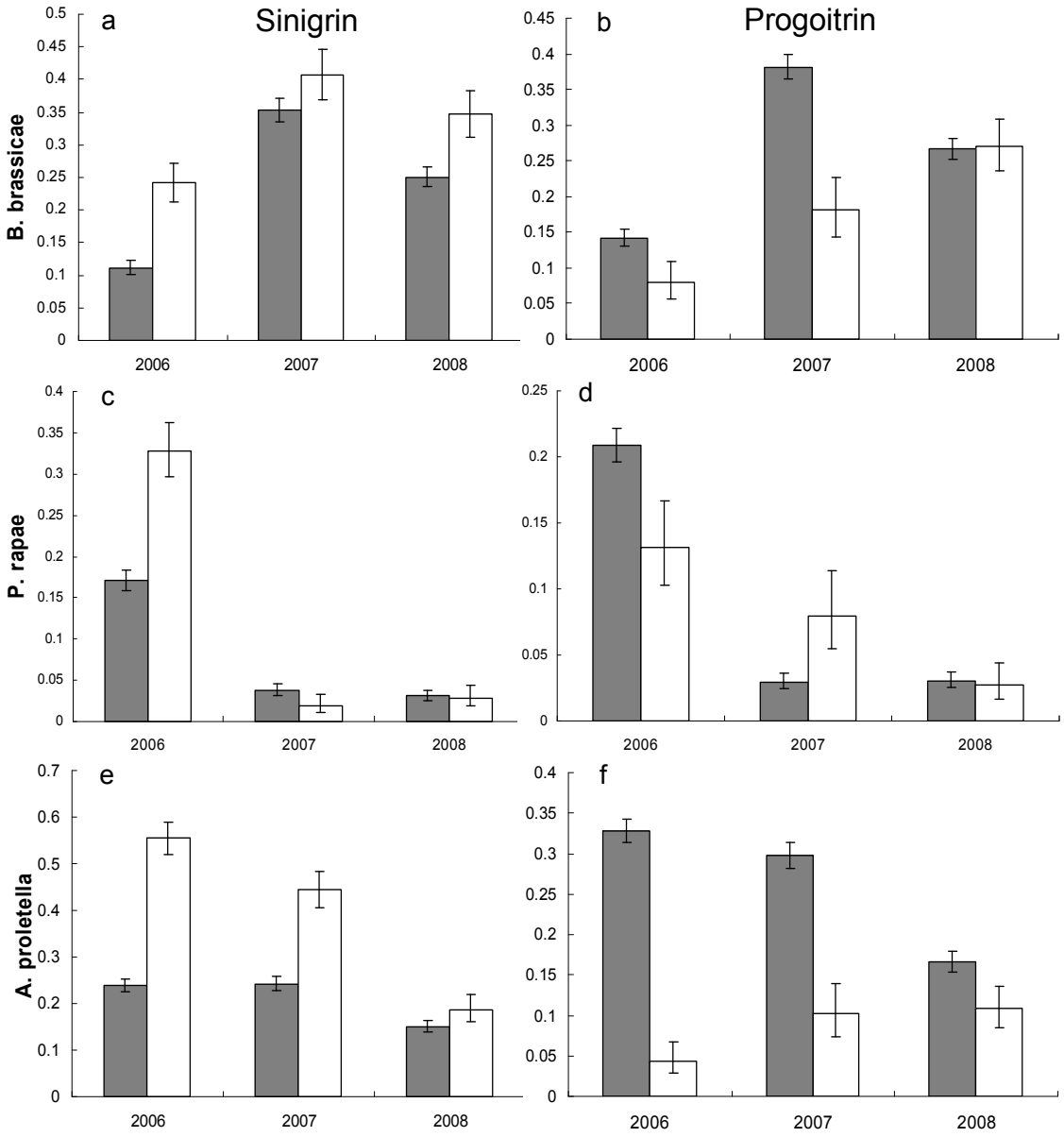

Figure 3.1 Responses of herbivore species to the presence or absence of sinigrin and progoitrin in plants within populations for the 3 study years. Mean probability of a plant lacking (white bars) or producing (grey bars) the glucosinolates sinigrin and progoitrin being infested with *B brassicae, P. rapae* and *A. proletella*. Means and standard errors calculated from a generalised linear model of herbivore response to individual glucosinolates across years for an average survey time, ignoring the random effects of county, population and plant ID. Hence the graphs show the raw, main effects of glucosinolate presence or absence.

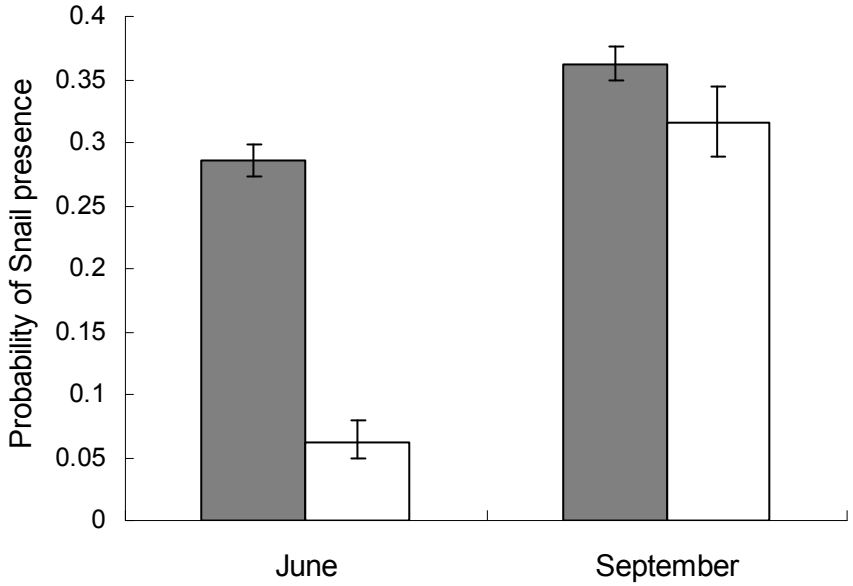

Figure 3.2. Mean probability of a plant lacking (white bars) or producing (grey bars) sinigrin being infested with snails during the survey periods for an average year**.** Means and standard errors calculated from generalised linear models of herbivore response to sinigrin, ignoring spatial structuring of county, population and plant ID. Hence the graphs show the raw, main effects of sinigrin presence.

Although fewer herbivores showed significant responses to progoitrin compared with the intra-population scale, effects were detected at the inter-population scale which were not significant within populations. Furthermore, the effects of sinigrin at this scale were more consistent: fewer interactions were found between the glucosinolate and year or survey which indicates that the processes structuring some of the species of herbivores between populations show less temporal variation.

At the inter-population scale, the proportion of plants infested with *B. brassicae* showed a significant negative response to the proportion of plants in a population producing sinigrin (Table 3.1, Fig. 3.3a). Year and survey timing also influenced cabbage aphid infestation rate (Table 3.1). Progoitrin did not affect the proportion of plants infested with *B. brassicae* (Table 3.1). Contrary to the intra-population results, *P rapae* was not affected by sinigrin or progoitrin at the inter-population scale: only year and survey timing influenced the proportion of plants infested with this lepidopteran (Table 3.1). The inter-population result for the response of *A. proletella* to sinigrin was similar to the effects of the glucosinolate at the intra-population scale: the infestation rate of whitefly is negatively correlated with the proportion of plants in the population producing sinigrin (Table 3.1, Fig. 3.3b). The response of whitefly to the proportion of plants producing sinigrin is not affected by year or survey time (Table 3.1). The proportion of plants producing progoitrin had no effect on infestation by *A. proletella* and showed no interaction with year or survey time (Table 3.1). The inter-population results for snails closely resemble the correlations found at the intra-population scale: the proportion of plants producing sinigrin positively affected snail infestation and this response varied with survey time (Table 3.1, Fig. 3.3c). The proportion of plants infested with snails also varied with survey time and year (Table 3.1).

Pieris brassicae presence showed no correlation with sinigrin or progoitrin within populations, but at the population scale the proportion of plants infested with this species was significantly correlated with both glucosinolates (Table 3.1): the proportion of plants producing sinigrin and the proportion producing progoitrin both had a positive effect on the infestation rate of *P. brassicae*. However, the interaction between sinigrin and progoitrin was negative meaning that the positive correlation between *P. brassicae* and both glucosinolates showed diminishing returns (Table 3.1). Survey time and year also influenced the proportion of plants infested with *P. brassicae* (Table 3.1).

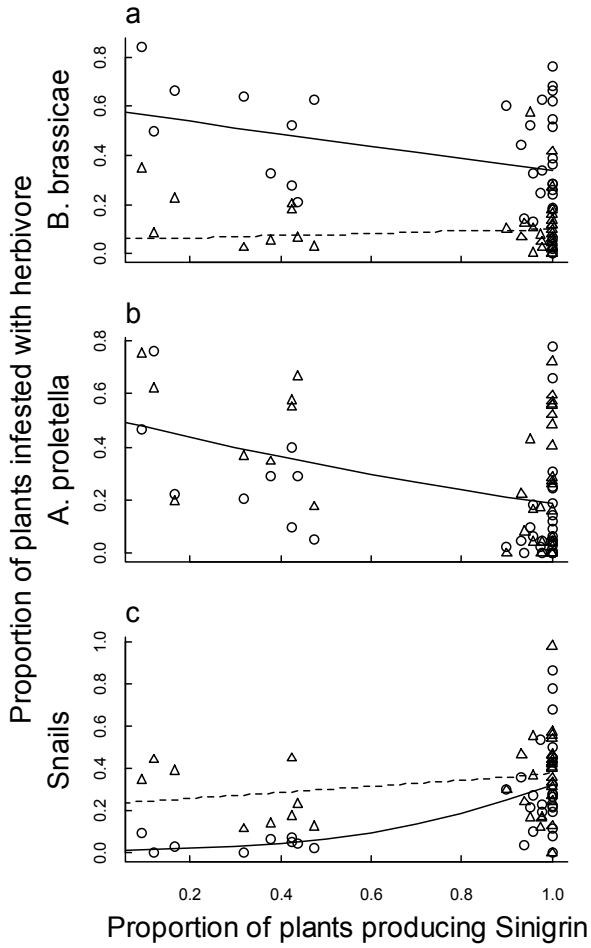

Figure 3.3. Herbivores showing significant responses to sinigrin at the inter-population scale. Results from June (circles and solid line) and September (triangles and dotted line) surveys are presented for herbivores showing a significant effect of survey time, (snails and *B. brassicae*). *A. proletella* showed a response to the main effect of sinigrin only, therefore, one fitted line is shown demonstrating the effect of sinigrin for the average survey and year (3.3c). Fitted lines are based on generalised linear models of herbivore responses (presence or absence) to sinigrin ignoring correlated influence of other glucosinolates and the random effect of population. Hence regression lines show the raw, main effects of glucosinolate frequencies, while significance indicators refer to mixed effects models. This allows fitted lines to fit the data visually, and avoids shrinkage of best linear unbiased predictors in maximum likelihood mixed effect models (Crawley, 2007).

Table 3.1 Significance of all fixed factors tested in the generalised linear mixed effects models for each herbivore. The fixed effects column and its corresponding *P*-value shows the significance of each main effect or interaction tested (main effects could only be tested if they were not involved in any significant interactions). The number of plants infested by each herbivore (n), the minimal adequate model (MAM; the simplest model that contained only significant fixed effects) and its overall significance (tested against the null model) as well as its false discovery rate-adjusted q-value are shown. The q-values control the scale-wide false discovery rate to have $\alpha = 0.05$ and so can be interpreted in the same way as conventional *P*-values.

Scale	Herbivore	n	MAM	Fixed effects	*P*-value	MAM vs. null (*P*-value)	MAM vs. null (*q*-value)
Intra-population	*B. brassicae*	2006:150 2007:327 2008:265	sinigrin*year + progoitrin*year + Survey	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year survey	0.17 0.83 0.35 0.04 0.01 <0.01	<0.001	<0.001
	P. brassicae	2006:57 2007:24 2008:56	year + Survey	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year survey sinigrin progoitrin year	0.70 0.33 0.33 0.23 0.81 <0.01 0.43 0.68 <0.01	<0.001	<0.001
	Snail	2006:361 2007:296 2008:243	sinigrin*survey + Year	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year year progoitrin	<0.01 0.84 0.15 0.26 0.26 <0.01 0.18	<0.001	<0.001
	P. rapae	2006:222 2007:31 2008:30	sinigrin*year + progoitrin*year + survey	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year survey	0.13 0.92 0.14 0.02 0.04 <0.01	<0.001	<0.001

Table 3.1 continued:

Scale	Herbivore	n	MAM	Fixed effects	*P*-value	MAM vs. null (*P*-value)	MAM vs. null (*q*-value)
Intra-population	*A. proletella*	2006:330 2007:251 2008:156	sinigrin*year + progoitrin*year + survey	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year survey	0.15 0.16 0.49 <0.01 <0.01 <0.01	<0.001	<0.001
Inter-population	*B. brassicae*	2006:150 2007:327 2008:265	sinigrin + year*survey	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year survey: year sinigrin progoitrin	0.06 0.39 0.13 0.16 0.19 <0.01 <0.01 0.71	0.002	0.002
	P. brassicae	2006:57 2007:24 2008:56	Sinigrin*progoitrin + year *survey	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year survey: year	0.47 <0.01 0.89 0.90 0.30 <0.01	0.001	0.002
	Snail	2006:361 2007:296 2008:243	sinigrin*survey + year*survey	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year progoitrin year: survey	<0.01 0.86 0.63 0.54 0.97 0.58 <0.01	<0.001	<0.001
	P. rapae	2006:222 2007:31 2008:30	year + survey	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year year: survey sinigrin progoitrin survey year	0.98 0.63 0.52 0.21 0.51 0.53 0.39 0.53 <0.01 <0.01	<0.001	<0.001
	A. proletella	2006:330 2007:251 2008:156	sinigrin	sinigrin: survey sinigrin: progoitrin progoitrin: survey sinigrin: year progoitrin: year year: survey sinigrin progoitrin survey year	0.63 0.13 0.50 0.14 0.31 0.90 <0.01 0.10 0.06 0.12	<0.001	<0.001

Discussion

The responses of five herbivore species to variation in the aliphatic glucosinolate profiles of their host plant were investigate at the intra- and inter-population scales over a three year period. Within populations, the herbivores *B. brassicae, A. proletella* and *P. rapae* responded to the presence of sinigrin and progoitrin, while snails showed a response to sinigrin but not progoitrin. At the inter-population scale, *B. brassicae, A. proletella* and snails showed a response to sinigrin, but not progoitrin, and *P. brassicae* infestation rate was correlated with sinigrin and progoitrin. The herbivore species varied in the direction of their responses to the glucosinolates.

INTER-ANNUAL VARIATION IN THE RESPONSES OF HERBIVORES

Possible ecological mechanisms for the differences in the responses of herbivore species to structural variation in glucosinolates are discussed in Chapter 2. Therefore, this discussion will focus on the inter-annual consistency in the responses to glucosinolates shown by the different herbivore species, and how these responses may play a role in the maintenance of glucosinolate variation in natural populations by top-down differential selection.

Brevicoryne brassicae showed consistent responses to sinigrin across both ecological scales. Within populations, aphids were less frequently observed on plants producing sinigrin. Although year had an effect on the response of *B. brassicae*, the overall direction of the response did not vary between years. *B. brassicae* also showed a negative response to sinigrin at the inter-population scale. Populations with a higher proportion of plants producing sinigrin had a lower aphid infestation rate. The responses of *B. brassicae* to sinigrin presence are identical to the results found in the analysis of the 2006 survey only. The response of *B. brassicae* to progoitrin was less consistent: the herbivore showed a positive response to progoitrin presence at the intra-population scale

but the extent of this response varied between years. Furthermore, no response of *B. brassicae* to progoitrin was detected at the inter-population scale. The responses of *B. brassicae* to progoitrin presented here contradict the results found for this herbivore in Chapter 2, which showed no effect of progoitrin on aphid presence within populations, but that responses of aphid infestation rates to progoitrin became evident at the larger ecological scale. A survey over three years is more statistically powerful and this may explain why finer scale responses of *B. brassicae* to progoitrin are detected in this chapter, however, the lack of an inter-population scale response is less intuitive. Overall, the responses of herbivores to progoitrin are weaker and less consistent than the results observed for sinigrin and this may be due to the lower variation in progoitrin presence or absence across populations compared to sinigrin. This is a constraint of surveying natural populations. The fact that responses to progoitrin are observed despite this caveat indicates that progoitrin production may indeed be involved in structuring some herbivore species. Conclusive evidence would require a more detailed investigation in an experimental set-up where the effects of progoitrin can be disentangled from the effects of other glucosinolates.

Snails showed a positive response to the presence of sinigrin that was highly consistent across both ecological scales. Within populations, plants producing sinigrin were more likely to be infested with snails, and this response was stronger in the early season survey. Similarly, snail infestation rate showed a positive correlation with the proportion of plants in a population producing sinigrin and the slope of this correlation was greater in the early season surveys. Year was significant but only as an additive effect and showed no interaction with sinigrin at either ecological scale. Snails showed no response to progoitrin within or between populations. These results are identical to the results presented in Chapter 2, indicating that the responses of snails to plant glucosinolate profiles are spatially and temporally consistent.

The presence of *P. brassicae* was not affected by sinigrin or progoitrin at the intra-population scale: the presence of this herbivore varied only with survey time and year. However, at the inter-population scale, both glucosinolates became important in determining the proportion of plants in a population infested by *P. brassicae*. Both glucosinolates had a positive effect on the proportion of plants infested with *P. brassicae*, but with diminishing returns. This response was also detected in the analysis of the 1st survey year (2006) but did not remain significant after FDR adjustment. The results indicate that *P. brassicae* is attracted to populations with higher proportions of sinigrin or progoitrin plants. This seems reasonable as the herbivore is a specialist on plants containing glucosinolates and previous studies have shown that sinigrin concentration has a positive effect on *P. brassicae* growth rate (Smallegange et al. 2007), However, the results presented here indicate that the attraction is reduced in populations that have high proportions of plants producing both aliphatic glucosinolates. Perhaps the combination of glucosinolates has a synergistic effect that reduces the palatability of plants to *P. brassicae.* Alternatively, there may be an increase in cost associated with the detoxification of a greater number of chemicals.

Pieris rapae presence on plants at the intra-population scale was affected by both sinigrin and progoitrin presence. The herbivore species showed substantial inter-annual variation in the direction of the response to both glucosinolates. From these results it is not possible to predict the response of *P. rapae* to either glucosinolate in any one year. There was no discernable effect of either glucosinolate on the infestation rate of *P. rapae* between populations: at this scale only survey timing and year affected the proportion of plants in a population infested with the herbivore.

It is also difficult to explain why responses of *P. rapae* to sinigrin and progoitrin were detected within, but not between populations; and why *P. brassicae* was found to respond to sinigrin between, but not within populations (particularly as the results in

Chapter 2 show that *P. brassicae* responds to sinigrin presence at the intra-population scale). A study by Moyes and Raybould (2001) on wild cabbage populations in Dorset identified a significant population scale correlation between the mean concentration of gluconapin (3-butenyl glucosinolate) and the proportion of cabbage seed pods infested with seed weevil (*Ceutorhynchus assimilis*): populations with a higher mean concentration of gluconapin had a higher proportion of pods infested with seed weevil larvae. There was no evidence of a correlation within populations leading to the conclusion that the herbivore is attracted to patches of plants with high concentrations of gluconapin, but does not discriminate between plants within these patches. However, population-scale conclusions drawn from a study which included only four populations can only be accepted with caution as one population could drive a spurious correlation. In the case of the responses of the *Pieris* species in this study it is possible that *P. brassicae* responds to coarse scale chemical signals but shows no preference or is not able to distinguish between plants within populations; whereas *P. rapae* distributes itself according to fine scale variations in plant chemistry which do not scale up to population responses. However, it is more likely that this outcome is due to constraints in the survey methodology (discussed below): particularly as the results found for *P. brassicae* in the first survey year show a significant positive response to sinigrin within populations but no significant response (after FDR correction) between populations. Furthermore, both species have specialist chemoreceptors on the foreleg tarsi which allow adults to identify different deterrent plant compounds (van Loon and Schoonhoven 1999).

Within populations, *A. proletella* responded to sinigrin and progoitrin presence. There was evidence of inter-annual variation in the strength of the responses, but the herbivore still showed a consistent, positive response to progoitrin and a consistently negative response to sinigrin. At the larger spatial scale, the infestation rate of *A.*

proletella between populations is affected only by the proportion of plants in the population producing sinigrin. Thus, the negative effect of sinigrin on *A. proletella* presence observed within populations is retained at a larger scale, and the response at this scale is not affected by survey timing or year.

Overall, the results of the three year survey correlate well with the responses of herbivores detected in the 1st survey year (presented in Chapter 2). The abundance of herbivore species varied between years but in general the numbers of insects were greatest in 2006: this year was much warmer and drier than the subsequent years which may explain the inter-annual variation in herbivore numbers.

The three year survey revealed an additional preference of *A. proletella* for plants lacking sinigrin within populations which was not observed in the first survey year. These herbivore species have passive (wind dispersal in the case of *B. brassicae* and *A. proletella* (Compton 2002)) or limited dispersal mechanisms. Yet the preferences of the herbivores to the presence or absence of sinigrin scale-up to a response to the frequency of phenotypes between populations. This suggests a strong selection pressure of plant chemistry on herbivores that acts across both ecological scales. In addition, a greater number of herbivore species showed responses to progoitrin: *B. brassicae, A. proletella, P. brassicae* and *P. rapae*.

The three species that showed the most consistent responses to glucosinolates across both scales were also the most abundant herbivores: *B. brassicae*, *A. proletella* and snails. The survey technique used is only a snapshot of herbivore presence, and may not be sufficient to give a clear picture about the responses of the less abundant herbivore species. The methodology used is sufficient to provide a strong indication that structural variation in glucosinolates may be responsible for structuring the herbivore community across a wide range of species. However, more detailed surveys and experiments are required in order to provide more information on the responses of

certain species of herbivores such as Lepidoptera: which are present in lower abundance but cause extensive damage to plant tissues. In addition, the survey methodology used did not control for the genetic background of the herbivores. The Pierids are highly mobile and can migrate large distances (Richards 1940; Feltwell 1982) therefore the insects observed in the populations may come from different genetic stock each year and this may explain the more variable responses to glucosinolates show by these species compared to herbivores with more limited dispersal mechanisms.

IMPLICATIONS FOR THE MAINTENANCE OF SECONDARY METABOLITE DIVERSITY

For top-down differential selection by herbivores to influence the maintenance of the observed inter- and intra-population variation in glucosinolate phenotypes, herbivore species must demonstrate different responses to genetically determined plant defence chemical variants, and herbivore abundance should also vary across populations or over time. We previously detected significant differential responses of herbivore species to qualitative variation in plant defence compounds over a one year survey period and surmised from these results that plant chemistry can structure the herbivore community (Chapter 2). The results from an extended survey over three years show that several of the herbivore species showed highly consistent responses across years and at both of the ecological scales investigated. In addition, we observed fluctuations in the abundance of herbivores between years and surveys. Evidence that herbivore attack has differential fitness effects on plants varying in chemical phenotype is required to provide further verification for the role of herbivores in the maintenance of glucosinolate diversity.

Acknowledgments

Thanks to J. Blount, A. Barlow and N. van Dam for providing advice on HLPC and techniques for purifying desulphoglucosinolates, Matthias Wichmann for advice about the Dorset sites, Isobel Giblin and James Grecian for fieldwork assistance. P. Behrens, T. Jones and Torbay Coast and Country Side Trust allowed access to field sites.

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Chapter 4

Bottom-up effects of glucosinolate variation on aphid colony dynamics in wild cabbage populations

Published in Ecological Entomology

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Abstract

1. There is an ongoing debate about the relative importance of top-down and bottom-up regulation of herbivore dynamics in the wild. Secondary metabolites, produced by plants, have negative effects on survival and growth of some herbivore species, causing bottom-up regulation of population dynamics. Herbivore natural enemies may use plant secondary metabolites as cues to find their prey, but their survival and reproduction can be influenced by the upward cascade of secondary metabolites through the food web. Thus plant chemistry might also affect herbivore populations by mediating top-down regulation.

2. We investigated the influence of heritable variation in aliphatic glucosinolates, a class of secondary metabolites produced by *Brassica* plants, on the relative importance of top-down and bottom-up regulation of *Brevicoryne brassicae* (mealy cabbage aphid) colonies in natural *Brassica oleracea* (wild cabbage) populations. We manipulated the presence of natural enemies on plants differing in their glucosinolate profiles, and monitored aphid colony growth and disperser production.

3. Aphid colony sizes were significantly smaller on plants producing sinigrin, compared to plants producing alternative aliphatic glucosinolates. Aphid natural enemy numbers correlated with aphid colony size, but there was no additional effect of the plants' chemical phenotype on natural enemy abundance. Furthermore, experimental removal of natural enemies had no effect on aphid colony size or production of winged dispersers.

4. Our results provide evidence for glucosinolate-mediated, bottom-up regulation of mealy cabbage aphid colonies in natural populations, but we found no indication of top-down regulation. We emphasise that more studies of these processes should focus on wild communities.

Key-words:

Brassica oleracea, *Brevicoryne brassicae*, bottom-up effects, natural enemies, plant-insect interactions, top-down effects

Introduction

Phloem-feeding herbivores such as aphids cause very little external damage to plants, but can have a highly detrimental impact on plant fitness. Aphids are vectors of plant diseases (Dixon 1998; Walling 2008). In addition, their short development times and parthenogenetic reproduction can result in heavy infestations (Goggin 2007) which deplete nutrients and photosynthetic compounds. However, plants are not defenceless against aphids: despite the minimal tissue damage, aphid attack can be detected by plants (de Vos et al. 2007) leading to the initiation of a number of resistance mechanisms (Thompson and Goggin 2006; Smith and Boyko 2007).

Plant secondary metabolites play an important role in limiting aphid infestation, for example by reducing their intrinsic rate of increase or repelling them through production of certain volatiles (Cole 1997; Goggin 2007; Smith and Boyko 2007). Additionally, the effects of secondary metabolites may be mediated by higher trophic levels: volatile secondary metabolites can attract aphid parasitoids (Bradburne and Mithen 2000; Kessler and Baldwin 2001; Blande et al. 2007) and some species can differentiate between host-infested, non-host infested and uninfested plants (De Moraes et al. 1998; Du et al. 1998; Rose et al. 1998; van Poecke et al. 2003; Girling et al. 2006). Variation in secondary chemistry might therefore be expected to have a profound effect on plant quality as a food source, and influence both top-down and bottom-up regulation of herbivores (Hunter 2003; Ode 2006). However, the relative importance of direct effects of secondary metabolites (bottom-up effects) compared to natural enemies

(top-down effects) in the regulation of herbivore populations is a contentious topic (Hunter and Price 1992; Walker and Jones 2001).

The widespread use of biological control in agriculture indicates a general belief that top-down effects are important in limiting herbivore infestation (Hairston et al. 1960; Agrawal 2000). However the complexity of natural communities could buffer these top-down effects (Hunter and Price 1992). When attempting to elucidate the relative roles of bottom-up and top-down factors in governing herbivore dynamics, it is important to test the conclusions from experiments in simplified laboratory or agricultural systems with studies in complex natural communities (Ode 2006; Gols et al. 2008).

As part of a research programme investigating ecological and evolutionary mechanisms that maintain diversity in the chemical phenotypes of wild cabbage *Brassica oleracea* (*Brassica oleracea* L. var. *oleracea*, subsequently referred to as *B. oleracea*), we performed surveys and experiments to determine links between plants' defence metabolites and the regulation of cabbage aphid colonies. Mealy cabbage aphid (*Brevicoryne brassicae*) colonies are found less frequently on plants producing the glucosinolate sinigrin compared to plants with alternative aliphatic glucosinolate profiles (Newton et al. 2009). We therefore predicted that aphid colonies on sinigrin-producing plants would be smaller and produce fewer dispersers than colonies on plants lacking sinigrin. To test this we compared aphid colony dynamics on plants differing in glucosinolate profile in natural populations of wild cabbage plants. Then, in order to investigate the relative importance of glucosinolate-mediated, bottom-up and top-down effects on aphid colony dynamics, we performed experimental manipulations of aphid natural enemies in this wild system.

Brassica oleracea produces aliphatic glucosinolates, a class of secondary metabolites involved in herbivore defence (Li et al. 2000; Wittstock et al. 2004), which has well characterised genetics (Magrath et al. 1994; Parkin et al. 1994; Mithen et al. 1995a; Gao et al. 2007). Glucosinolates are stable and non-volatile when intact, but upon damage to plant tissue they are broken down by myrosinase, a ß–thioglucosidase enzyme, and converted to volatile end products which show varying degrees of toxicity to herbivores (Li et al. 2000; Wittstock et al. 2004).

Large infestations of *Brevicoryne brassicae* (mealy cabbage aphid) can be observed in wild *B. oleracea* populations during the flowering period (April-June in the UK). Perennial wild cabbage is a primary host plant for early-season infestations of *B. brassicae*, an important specialist pest of Brassica crops (Cole 1997), and may be the source of many crop infestations later in each growing season. The *B. brassicae*- *B. oleracea* system may represent a closely coevolved interaction: although aliphatic glucosinolates can influence the presence of *B. brassicae* in the wild (Newton et al. 2009) the aphids are able to sequester glucosinolates from their food plant and use them in defence against their own natural enemies by producing a form of myrosinase enzyme (Bridges et al. 2001; Kazana et al. 2007; Pratt et al. 2008).

B. brassicae are commonly attacked by a variety of natural enemies, including hoverflies (Diptera: Syrphidae) and the endoparasitoid *Diaeretiella rapae*, which has been shown to respond to volatiles produced by aphid-plant complexes (Girling et al. 2006). Due to the sequestration of glucosinolates by aphids, endoparasitoids and predators in the third trophic level will be directly exposed to phenotypic variation in the glucosinolates stored in the tissues of their host. Thus there is scope for the effects of glucosinolates to cascade up to higher trophic levels (Gols et al. 2008a; Gols et al. 2008b).

Structural variation in aliphatic glucosinolates is controlled by five loci: *GSL-pro* and *GSL-elong* control the length of the variable side chain producing propyl- and butyl-derived glucosinolates respectively. Three additional loci (*GSL-sulph*, *GSL-alk* and *GLS-oh*) control further modification of side-chain structure (Magrath et al. 1994; Parkin et al. 1994; Mithen et al. 1995a). All wild cabbage plants in this study produced the full range of butyl glucosinolate derivatives synthesised by gene products from the *GSL-elong, GSL-sulph*, *GSL-alk* and *GLS-oh* loci. The variation in glucosinolate profiles across all plants tested was caused by presence or absence of sinigrin glucosinolate, controlled by gene products from the *GSL-pro* locus, giving two phenotypes (plants producing butyl-derived aliphatics only, and plants producing butyls and sinigrin) across the three plant populations studied.

In this study we investigated the response of cabbage aphids to natural variation in the presence or absence of individual aliphatic glucosinolates, and not to quantitative variation in glucosinolate concentrations. This is because the presence or absence of aliphatic glucosinolates is a consistent form of variation between plants in the field and is a well studied, and well understood, heritable component of cabbage defence. We recognise that quantitative differences in glucosinolate concentration may influence aphid responses, however such concentrations can vary dramatically between plant tissues and through time, and are induced by herbivory (Bodnaryk 1992), rather than being a consistent, constitutive defence. Furthermore, the heritability of aliphatic glucosinolate concentrations is less well understood than the simple genetics underlying variation in their chemical structure. For similar reasons, we did not study indole glucosinolates, whose presence or absence in plants is induced by herbivory itself, and whose genetics are less well understood. Natural populations of *B. oleracea* tend to be very persistent (Wichmann et al. 2008) and individuals show inter- and intra-population variation in aliphatic glucosinolates (Mithen et al. 1995b; Moyes et al. 2000), providing

sufficient variation in this aspect of antiherbivore chemistry to allow powerful experiments and surveys. Furthermore, Newton et al. (2009) found that variation in herbivore attack on *B. oleracea* plants and populations were linked strongly to presence/absence patterns of aliphatic glucosinolates.

Materials and Methods

FIELD SITES

Flowering *B. oleracea* plants at three sites; PC1 (50°10'N, 5°42'W), PC2 (50°12'N, 5°42'W), and PC3 (50°10'N, 5°41'W) were used in the study. All three sites are located within a 5km stretch of coastline consisting of several sheltered coves on the south coast of Cornwall in south-western England. There is little variation in aspect or altitude between sites. Plant gene flow between the sites is highly probable: the coastline is popular with walkers which could lead to long distance seed dispersal (Wichmann et al. 2009) and pollen flow is likely as distance between the sites is less than the foraging range of many bee species (around 10km) (Visscher and Seeley 1982; Pasquet et al. 2008). A transect was laid at each site along which plants were selected at random and marked using individually-numbered copper tags. The basal stem diameter was measured for each marked plant as a standard measure of plant size (e.g. Stokes et al. 2004), and therefore as a potential predictor of aphid colony size. The transect start and end points were recorded using a GPS receiver. The field experiment was carried out during the flowering seasons in May-June 2007 and 2008.

GLUCOSINOLATE EXTRACTION AND ANALYSIS

Following Newton et al. (2009), one intact leaf was removed from each marked plant to determine the glucosinolates present. The leaf was boiled for 3 min on a gas camping stove (Coleman) immediately after removal from the plant to inhibit the action

of the myrosinase enzyme, which would otherwise hydrolyse the glucosinolates (Prestera et al. 1996). The leaf samples were then returned to the laboratory, frozen and freeze-dried to constant weight. The glucosinolates were extracted and converted to desulphoglucosinolates according to published methods (Graser et al. 2000; van Dam et al. 2004). Freeze-dried plant material was ground to a coarse powder and 100mg placed into a ventilated 1.7ml microtube. The glucosinolates were extracted with boiling 70% methanol and desulphated using sulphatase solution (prepared from Sigma-Aldrich type H-1 aryl sulphatase of *Helix pomatia*) on a DEAE Sephadex A-25 column (Sigma-Aldrich). The desulphoglucosinolates were eluted from the columns the following day. The samples were then freeze-dried and stored until required for HPLC.

The desulphoglucosinolates were separated using reverse-phase HPLC on a C18 Dionex column, with an acetonitrile - water gradient mobile phase (Spinks et al. 1984). The eluted desulphoglucosinolates were detected at 226nm using a Dionex PDA-100 Photodiode Array Detector. The glucosinolates were identified using sinigrin, gluconapin, progoitrin and glucoraphanin as external standards. The four aliphatic glucosinolate standards were converted to desulphoglucosinolates on Sephadex columns as described above for plant extracts. The retention times of peaks were also compared with published retention times (Spinks et al. 1984; Mithen et al. 1995; Kiddle et al. 2001).

EXPERIMENTAL DESIGN

We carried out experiments on *B. oleracea* plants during the flowering season: a time when aphids can be found in high numbers on the flowering stems. There is evidence that flowering stems have high levels of antiherbivore protection, perhaps because damage to the reproductive parts of the plants may have high fitness costs. For example *Arabidopsis* flowering stems contain cells near the phloem which have high

concentrations of glucosinolates (Koroleva et al. 2000). Although *Arabidopsis* tends to flower in the spring before the majority of specialist species emerge (Harvey et al. 2007) if the presence of these cells in near the phloem is conserved across plant species this may be a specific defence strategy against aphid attack.

In April 2007, *B. brassicae* colonies were initiated on plants by adding 5 aphids to a developing flower stem (total number of study plants= 39), or, if an aphid colony was already present, aphids were removed until 5 remained. While this approach could have resulted in small biases in colony performance, we note that all colonies grew successfully and there was no bias in colony initiation method between plant glucosinolate phenotypes. The added aphids were sourced from pool collected from at least 10 randomly chosen colonies within the same cabbage population as the experimental plants. The colonies were allowed to develop and the numbers of unwinged, winged and parasitised aphid mummies, as well as all aphid natural enemies, on the entire plant were recorded every three days until the end of the flowering period in June.

In 2008, aphid colonies were initiated on plants (n = 65) as in 2007. In order to standardise the initiation of aphid colonies, any aphids present were removed twice a week from all plants using the same protocol for three weeks prior to the start of the experiment in April 2008. Several of the plants studied in 2007 were flowering again and were included in the study for a second year, but the majority of plants were unique to the 2008 study. Two natural enemy treatments were also applied to the colonies in a factorial design with plant sinigrin phenotype: half of the plants of each phenotype were assigned to the control treatment and the remainder were assigned to the natural enemy removal treatment. In the control treatment all aphid natural enemies were allowed to attack the colonies unhindered, while in the natural enemy removal treatment all parasitised aphids and aphidophagous insects were removed from the colonies using

fine forceps throughout the survey period. Subsequently, and until the end of flowering in June, plants were visited every three days to record the numbers of unwinged, winged and parasitised aphids as well as numbers of aphid natural enemies on plants in the control treatment and to remove aphidophagous insects and parasitised aphids.

STATISTICAL ANALYSIS

All statistical analysis was carried out using R 2.7.0 (2008). Two methods were used to analyse the aphid colony dynamics. First, we analysed the dynamics of colonies exposed to natural enemy attack in 2007 and 2008 using a generalised linear mixed effects model with Gaussian errors to test the effect of plant phenotype on unwinged, winged and parasitised aphid counts through time, and the proportion of winged and parasitised aphids per visit. All counts of aphids and parasitoids were log-transformed to standardise and homogenise residuals. We accounted for repeated sampling of aphid colonies through time and spatial structuring of plants by modelling year, plant population and plant identity as random effects. We also accounted for repeated measures of each aphid colony through time: recognising that aphid colonies grow and then decline, we introduced the linear and quadratic effects of date to the random effects structure and allowed these regression coefficients to vary independently for each plant. The fixed effect of plant phenotype on aphid colony size, disperser production and natural enemy attack was then tested for significance using model simplification via likelihood ratio tests of maximum likelihood versions of the models (Crawley 2007).

We used the same mixed modelling approach to analyse the natural enemy removal experiment in 2008. The effect on colony size of natural enemy removal, plant phenotype and their interaction (fixed effects), on all colonies studied in 2008 was tested using a linear mixed effects model with Gaussian errors and an identical random effect structure to that used in the control treatment colony dynamics analysis.

In the second method, the aphid count data for each colony was simplified into peak number (i.e. the maximum number of unwinged aphids observed at a single time point) and cumulative numbers (i.e. the total numbers of winged and unwinged aphids and natural enemies observed on each plant over the course of the study period). This simplification of aphid colony dynamics into derived variables (peak size and cumulative size) removed the need to consider repeated measures within the season, and allowed an additional analysis of the effect of hoverfly larvae, which were not present in high enough numbers for the first analysis but may have a large impact due to the high numbers of aphids each larva can consume. Cumulative numbers of hoverfly larvae and unwinged, winged, and parasitized aphids were analysed by generalised linear models with negative binomial errors. Plant phenotype, basal stem diameter and population were included as fixed effects in all models and cumulative numbers of unwinged aphids was added as a covariate in the hoverfly larvae, winged, and parasitised aphid analyses. Year was included in these models as a blocking factor, to absorb the effect of time and repeated measures on plants. The efficacy of parasitoid removal was also analysed using a generalised linear model with negative binomial errors. We analysed the cumulative numbers of parasitised aphids (numbers present in the control treatment and numbers removed in the removal treatment) as the response variable and treatment, while plant basal stem diameter and plant population were included as covariates. We tested significance of explanatory variables and their interactions in all analyses using model simplification and likelihood ratio tests (Crawley 2007).

Prior to these analyses, we confirmed a lack of spatial structuring of phenotypes within each site using a Mantel test (Mantel 1967) to investigate spatial autocorrelation between sinigrin phenotype and distance along the transect, which might have resulted in confounding effects of environmental stresses on aphid colony dynamics.

Results

EFFECT OF PLANT PHENOTYPE ON APHIDS

HPLC analyses confirmed that plant chemical phenotypes varied only in the presence or absence of sinigrin, i.e. all plants produced the full range of butyl-derived aliphatics, the absence of sinigrin was determined by a lack of a peak at the expected retention time for the glucosinolate (confirmed by standards of pure sinigrin). The sites showed slight differences in the frequencies of the two glucosinolate phenotypes (frequency of plants producing sinigrin in addition to all butyl-derived aliphatics: PC1 = 0.14, PC2 = 0.41, PC3 = 0.32): phenotype frequencies for the sites was determined from a minimum of 55 flowering and non-flowering plants. Results from the Mantel tests showed that there was no spatial autocorrelation in phenotype distributions along transects in each population (PC1 $P = 0.36$, PC2 $P = 0.57$, PC3 $P = 0.55$).

Aphid colony size was significantly influenced by plant phenotype: the number of unwinged aphids through time was consistently lower on plants with the sinigrin phenotype, compared to plants producing only butyl-derived glucosinolates ($\chi^2_1 = 4.080$, $P = 0.043$, Fig. 4.1). This result is supported by other measures of aphid colony size: cumulative number of unwinged aphids ($\chi^2_1 = 8.722$, $P = 0.003$, sinigrin plants: mean 2007 = 392; mean 2008 = 440, butyls only: mean 2007 = 3432; mean 2008 = 683), peak number of unwinged aphids ($\chi^2_1 = 7.772$, $P = 0.005$, sinigrin plants: mean 2007 = 105, mean 2008 = 112, butyls only: mean 2007 = 1004, mean 2008 = 195). Year also had a significant effect on aphid colonies ($\chi^2_1 = 10.671$, $P = 0.001$): fewer aphids were observed in 2008. Plant size (basal stem diameter), tested as a covariate, had no significant effect on the cumulative number of aphids ($\chi^2_1 = 2.615$, $P = 0.106$) or the number of winged dispersers ($\chi^2_1 = 0.292$, $P = 0.589$). There was no difference between phenotypes in the numbers of winged dispersers produced through time ($\chi^2_1 = 0.366$, $P = 0.545$), nor in the proportion of winged aphids produced by the colony at each time

point (χ^2_1 =0.627, P =0.429, Fig. 4.2). Although the cumulative number of winged aphids produced by colonies was affected by phenotype (χ^2_1 = 4.900, P = 0.026, sinigrin plants: mean 2007 = 36, mean 2008 = 21, butyls only: mean 2007 = 59, mean 2008 = 41), when cumulative number of unwinged aphids was added as a covariate in order to control for colony size the effect of plant phenotype disappeared (χ^2_1 = 1.911, P =0.167). Thus the apparent difference in disperser production between plant phenotypes was a simple numerical response to aphid colony size.

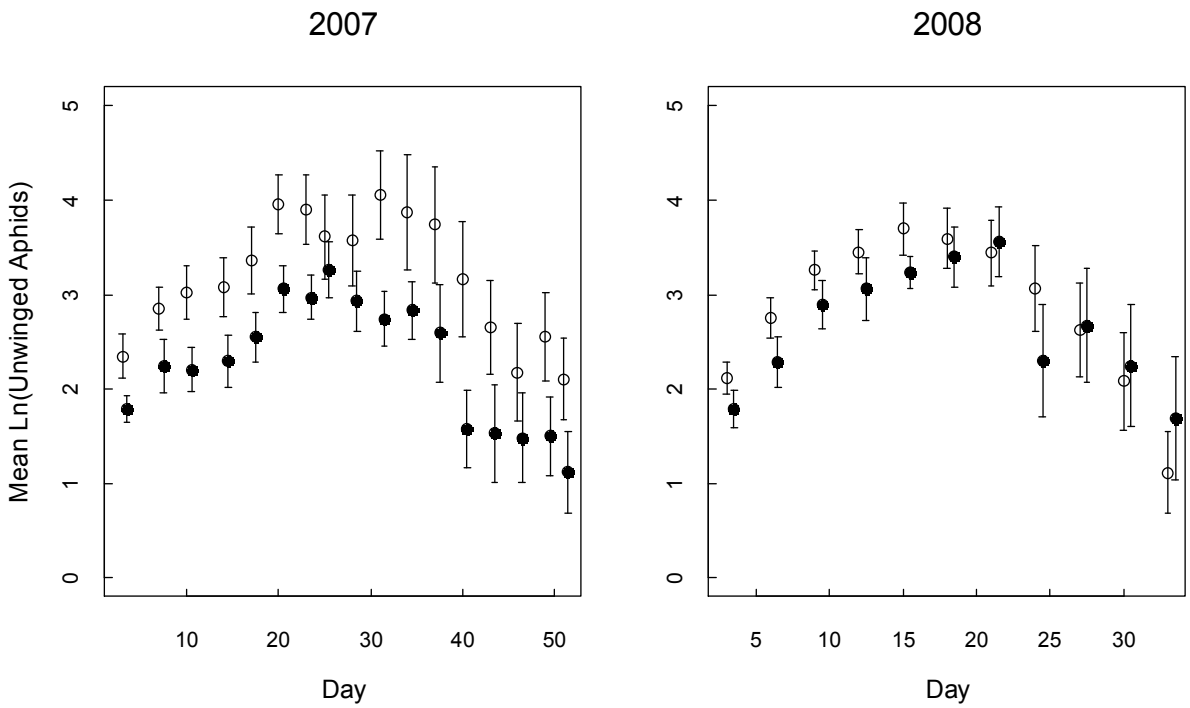

Figure 4.1. Mean counts of unwinged aphids (natural log scale) on plants producing (black circles) and lacking (open circles) sinigrin in 2007 (total number of study plants = 39) and 2008 (total number of study plants = 65). Error bars show +/- 1 standard error, calculated from raw data. Points for sinigrin have been shifted 0.5 days along the x axis for clarity.

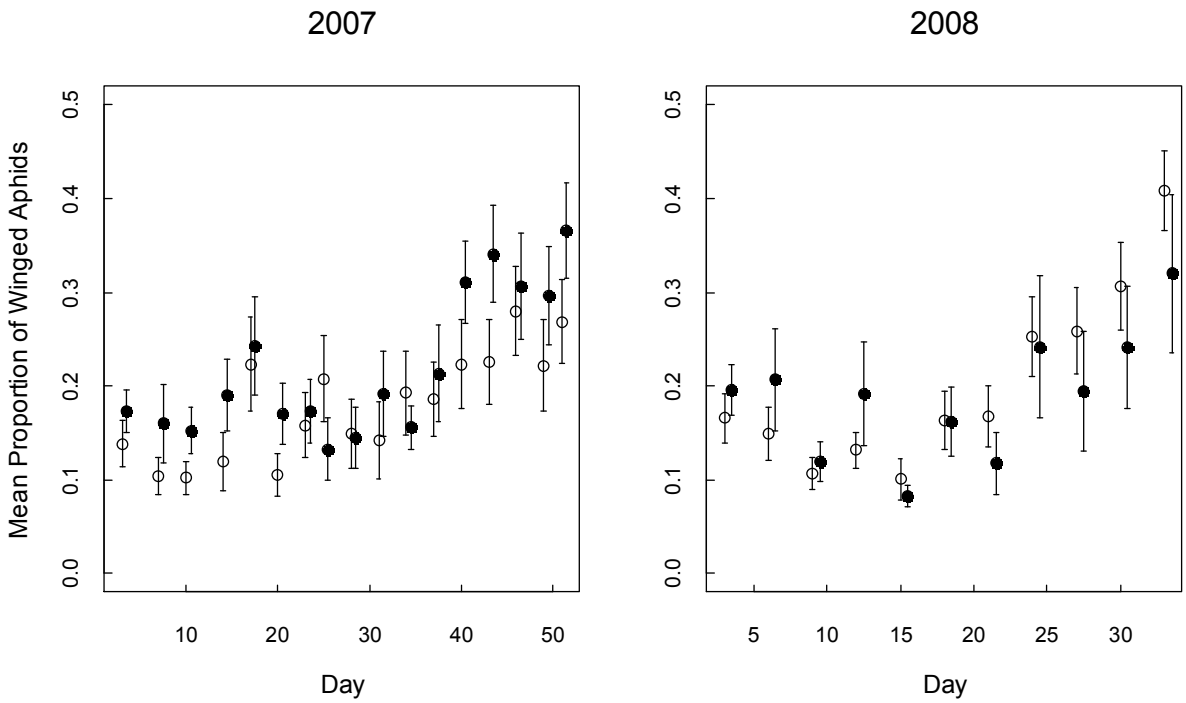

Figure 4.2. Mean proportions of winged aphids in colonies on plants producing (black circles) and lacking (open circles) sinigrin in 2007 and 2008. Error bars show +/- 1 standard error, calculated from raw data. Points for the sinigrin phenotype have been shifted 0.5 days along the x axis for clarity.

The major groups of natural enemies observed on the colonies were the aphid parasitoid *Diaeretiella rapae* and larvae of two species of hoverfly. Lacewing adults, eggs and larvae (Chryopidae, total number observed of all life stages = 4), *Araniella curcurbitina* (green orb spider, total = 3) and *Araneus diadematus* (garden cross spider, total = 60) were also observed. The number of *D. rapae*-parasitised aphids showed a significant, positive correlation with plant basal stem diameter ($\chi^2_1 = 8.231$, $P = 0.004$). There were consistently fewer parasitised aphids through time on sinigrin plants, ($\chi^2_1 = 6.340$, $P = 0.001$). However, when we accounted for aphid colony size by analysing the proportion of parasitised aphids in the colony at each time point there was no significant difference between phenotypes ($\chi^2_1 = 0.436$, $P = 0.509$, Fig. 4.3). The cumulative numbers of hoverfly larvae across the entire season did not differ between phenotypes ($\chi^2_1 = 1.626$, $P = 0.202$, sinigrin plants: mean 2007 = 0.53, mean 2008 = 0.18, butyls only: mean 2007 = 0.58, mean 2008 = 1.61). In addition the numbers of hoverfly larvae were also highly correlated with the aphid colony size ($\chi^2_1 = 7.937$, $P = 0.005$).

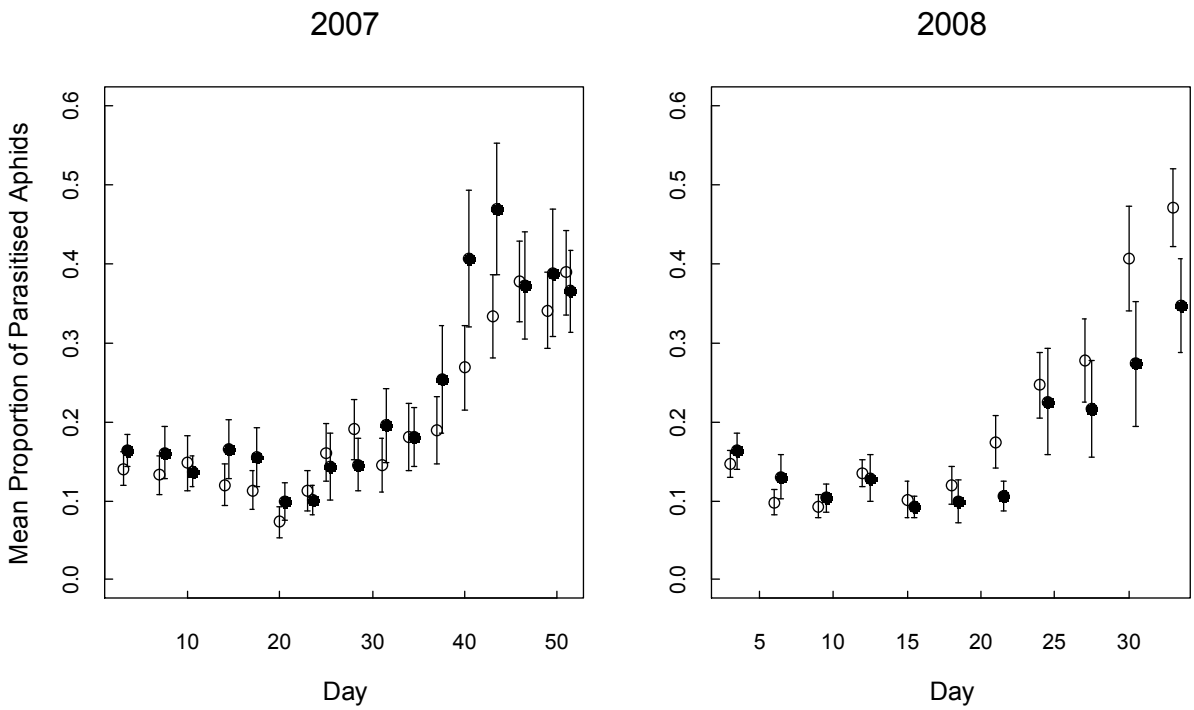

Figure 4.3. Mean proportions of *Diaeretiella rapae*-parasitised aphids in unmanipulated aphid colonies on plants producing (black circles) and lacking (open circles) sinigrin in 2007 and 2008. Error bars show +/- 1 standard error, calculated from raw data. Points for the sinigrin phenotype have been shifted 0.5 days along the x axis for clarity.

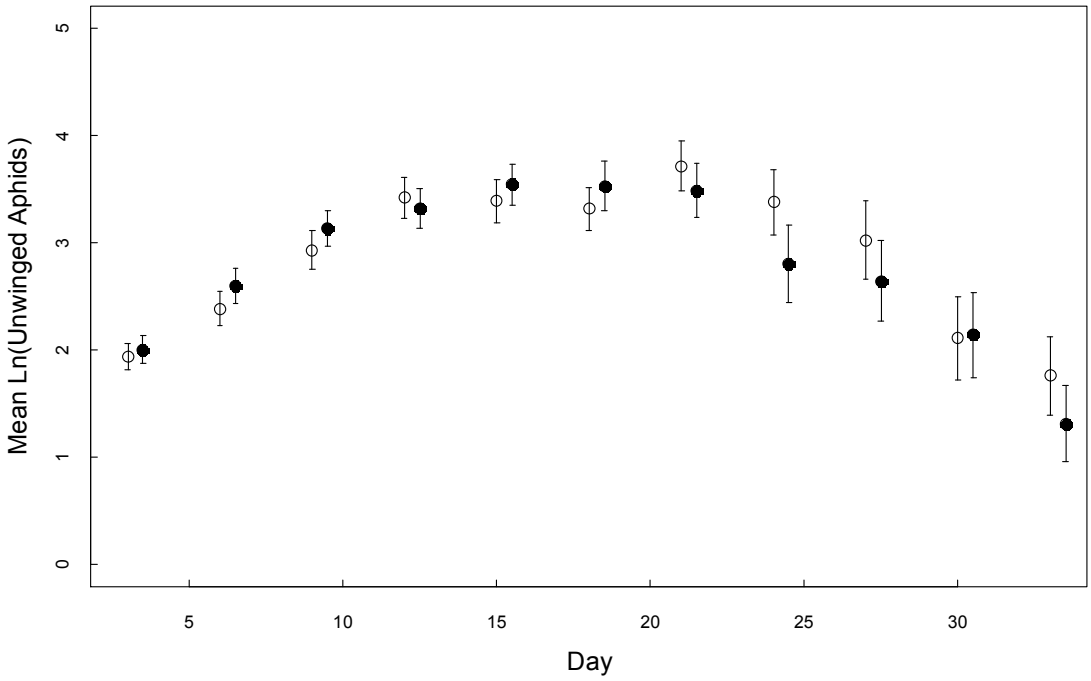

Figure 4.4. Mean counts of unwinged aphids (natural log scale) on plants in each treatment in 2008. Error bars show +/- 1 standard error, calculated from raw data. Closed circles represent control treatments and open circles represent the natural enemy removal treatment. For clarity the points have been separated by 0.5 days on the x axis.

The method of natural enemy removal in 2008 was effective, such that significantly more parasitised aphids were observed on aphid colonies in the control treatment than were removed from colonies in the natural enemy removal treatment (χ^2_1 = 13.176, P = 0.006, mean cumulative number of parasitised aphids removed per plant = 17, mean cumulative number present per plant in the control treatment = 43). Other natural enemy species had a slower growth rate and were easily identified and removed at the egg or early larval stage so their impact on the aphid colony was kept to a minimum (total number of natural enemy individuals (not including parasitised aphids): control = 129, numbers removed in the removal treatment = 106). Despite this, natural enemy removal had no significant effect on the numbers of unwinged aphids at each time point (χ^2_1 = 0.006, P = 0.802, Fig. 4.4) and showed no interaction with plant phenotype (χ^2_1 = 0.002, P = 0.965).

Discussion

The results presented here indicate that structural variation in *B. oleracea* glucosinolate profiles can have an impact on wild *B. brassicae* populations: colonies on sinigrin-producing plants were significantly smaller and produced fewer winged dispersers compared to colonies on plants in which the aliphatic glucosinolates had only butyl derived side-chains. This impact was not mediated by effects of sinigrin on aphid natural enemies, which showed only a numerical response to aphid numbers. Nor did removal of natural enemies affect aphid numbers. Thus, the results suggest that, in this wild system, bottom-up regulation of aphids may be more important than top-down effects.

We studied these processes in the wild using a mixture of survey and experiment, therefore the exact mechanisms could not be determined unequivocally. Many plant-insect studies are performed in the laboratory and these are important for

clarifying interactions or revealing cause and effect. It is important, however, that studies are carried out in natural populations, as a signal detected in a controlled environment may not be influential in the wild. Thus, even though the methods employed here were unavoidably less precise when compared to laboratory methods (we clarify these caveats in the discussion to follow), studies in natural populations are required to determine the importance of secondary metabolite variation for plant-insect interactions.

DIFFERENTIAL EFFECTS OF GLUCOSINOLATES ON APHID COLONY DYNAMICS

Although aphids cause very little external damage to plants, they are exposed to plant defence mechanisms (de Vos et al. 2007) and use a number of strategies to avoid plant defences (Walling 2008). Despite the minimal wounding caused by aphid attack we found that aphid colony sizes are positively correlated with the absence of sinigrin glucosinolate. This could be because damage by other herbivore species mobilises the toxins in the phloem. Alternatively, the negative effect of sinigrin on cabbage aphids may be due to a greater cost of sequestering this glucosinolate, although there is no published study of the costs of sequestering different glucosinolates.

Although sinigrin has been shown to promote aphid feeding (Wensler 1962; Nault and Styer 1972) it appears that the presence of this chemical has a negative effect on aphid colonies in the field, relative to alternative aliphatic glucosinolates. This result contributes to a surprisingly small literature supporting the hypothesis that aphid colony dynamics are regulated by variation in heritable plant metabolites (Cole 1997; Tosh et al. 2003) and also corresponds to a previous study in which we found a strong negative correlation between cabbage aphid presence and sinigrin presence in wild *B. oleracea* at both inter- and intra-population scales (Newton et al. 2009). However, this negative relationship between aphid colony size and presence of sinigrin seems to contradict

previous results from a glasshouse study by Cole (1997) which found that the intrinsic rate of increase of *B. brassicae* was higher on plant species with higher concentrations of sinigrin. These conflicting results may be due to a fundamental difference between the studies: Cole (1997) investigated variation in glucosinolate concentration across plant species, whereas the current study was concerned with the effects of variation in the presence of sinigrin within a species. It may be that in comparing plant species, sinigrin concentration has a positive impact on *B. brassicae*, but when comparing the effect of the presence or absence of sinigrin within a plant species there can be a negative effect.

Alternatively, the observed effects of sinigrin on aphid colonies may ultimately be due to a trait that is correlated with sinigrin presence. For example, Cole (1997) showed that concentrations of progoitrin, an aliphatic glucosinolate that was present in all plants in our study, was positively correlated with the intrinsic rate of increase of *B. brassicae*. In addition, *B. brassicae* was also affected by phloem amino acid concentration (Cole 1997). We did not test for a correlation between amino acids and glucosinolates within a plant. The disadvantage of studying wild plants was that we could not produce a full profile of each plant's primary and secondary metabolism. Therefore we can only implicate sinigrin in the control of aphid colony size. However, inclusion of plant size in our analyses provided some control for correlations between plant phenotype and quality. We found no significant effect of plant size on aphid colony size or disperser production. The Mantel tests showed no spatial grouping of plants in terms of sinigrin phenotype, which also supports our interpretation that differences in aphid colony dynamics were due to plant chemical phenotypes rather than confounding environmental variables.

We also note that although the aphids tend to feed on the flowering stem, we used leaf tissue to determine the aliphatic glucosinolate profile. This disparity is

unlikely to affect our findings on the effects of glucosinolate presence or absence. Although concentration of glucosinolates can vary among plant tissues (Shelton 2005), the ability of a plant to synthesise a compound is consistent across all tissues and is under strong genetic control (Magrath et al. 1994; Parkin et al. 1994; Mithen et al. 1995a)

Despite these caveats, this study presents strong evidence that plant chemistry can exert bottom-up pressures on aphid colony sizes. These results do not, however, clarify whether regulation is mediated by direct effects of chemistry, or indirectly via the upregulation of natural enemy attack, Teasing apart these mechanisms required experimental removal of natural enemies.

NO EVIDENCE FOR DIFFERENTIAL TOP-DOWN REGULATION

Cabbage aphids are known to sequester sinigrin from their host plant and to use it in defence against their own natural enemies (Kazana et al. 2007; Pratt et al. 2008). However, our results indicate that the apparent negative effects of sinigrin on aphids are not mediated through aphid natural enemies. There was no effect of sinigrin presence or absence on the abundance of either parasitoids or hoverfly larvae, which were correlated with aphid colony size only.

In this study we did not test the effect of sinigrin presence or absence on parasitoid fitness or host preference. Instead we aimed to determine whether fitness impacts of foodplant chemistry on higher trophic levels, as have been identified in laboratory assays (Gols et al. 2008a; Gols et al. 2008b), scale up to significant impacts on herbivore population dynamics. If higher trophic levels can act as an extension of a plant's defences against herbivore attack (Price et al. 1980) it is necessary that the toxicity of plant chemistry to herbivores does not cascade upwards to cause a corresponding negative effect on parasitoids, as this could counter any top-down effects

on herbivore populations. However, such toxicity cascades have been found in several laboratory-based studies: the performance of parasitoids in response to glucosinolates is similar to that of their Lepidopteran hosts (Gols et al. 2008a; Gols et al. 2008b), while similar bottom-up cascades can even be detected up to the fourth trophic level (Harvey et al. 2003). The control of aphid colonies by predators and parasitoids may be further complicated by the buffering effects of intra-guild predation (Ode 2006). For example, the consumption of early stage parasitoids in aphids by hoverfly larvae can have a significant influence on the parasitoid population (Muller and Brodeur 2002). Given that trophic cascades can lead to predictions of increased, decreased or null regulation of herbivores by natural enemies, we note the critical importance of studies carried out in natural communities, to help clarify patterns of real effects.

The majority of studies presenting evidence for top-down effects have been carried out on crop plants (reviewed in Hunter (2002)) which have been subject to intense artificial selection. Such systems are far removed from the conditions in natural populations. For example, the density of hosts in the wild will be substantially lower compared to crops in monoculture and this could decrease the foraging efficiency of aphid natural enemies (Gols et al. 2005). This raises the important question of whether the interactions identified in laboratory bioassays, such as chemically-mediated signalling between plants, herbivores and natural enemies, or links between plant chemistry and natural enemy fitness, scale up to population-level impacts in the wild.

Despite laboratory studies showing that parasitoid (Bradburne and Mithen 2000; Blande et al. 2007) and hoverfly (Verheggen et al. 2008) females respond to host-induced plant volatiles and can affect plant productivity through top-down effects (Stiling and Moon 2005), we found no direct effect of sinigrin on natural enemy abundance. Indeed we found no evidence of herbivore regulation by natural enemies at

all: relieving *B. brassicae* colonies from predation pressure had no effect on their overall size or the number of dispersers produced on either plant phenotype.

WHY NO EVIDENCE FOR TOP-DOWN REGULATION?

The apparent inability of parasitoids and hoverflies to regulate aphid hosts in this system, despite high levels of attack (around 50%), is surprising. It is possible that the effectiveness of parasitoids and predators may be reduced due to their longer generation times compared to aphids (Dixon 1998). Alternatively, as aphid colonies are relieved from natural enemy pressure other density-dependent mortality factors may become important, e.g. resource limitation (Dixon 1971; Dixon 1998). It is also possible that the act of removing parasitised aphids had a negative effect on the aphid colony which outweighed the effect of natural enemies, for example via the release of alarm pheromones or an energetic cost of aphid defensive behaviours (Nelson et al. 2004). Furthermore, despite results indicating that the method of natural enemy removal used in this study had a significant impact on levels of parasitism, total relief from natural enemy pressure was not achieved. Therefore, if the regulation of aphid colonies by parasitoids operates in a density-dependent manner there may have remained sufficient parasitoids to have an impact on aphid colony dynamics and lead to a non-significant result. Additionally, only late stage parasitoids could be removed because it is only possible to discern infested aphids once they form a mummy shortly before the emergence of adult parasitoids. Therefore the removal of late stage parasitoids will only reduce their negative impact on aphid growth and reproductive rate by preventing emerging adults from attacking aphids in their natal colony (Tang and Yokomi 1996) .

Although a large literature exists on the ability of parasitoids to use host-infested plant volatiles to locate their prey (De Moraes et al. 1998; Du et al. 1998; Rose et al. 1998; van Poecke et al. 2003; Girling et al. 2006), there is also evidence that some

parasitoid species are only able to distinguish between damaged and undamaged plants (Shiojiri et al. 2001). An additional complication is that the volatiles elicited by an herbivore species varies with plant species (Dicke 1999) making the detection of individual species by generalist parasitoids a complicated process (Hunter 2002). The fact that we found a greater number of parasitoids on plants with a larger basal stem diameter suggests that parasitoid foraging in this system may rely on cues that are less sophisticated than variation in plant defence compounds. We are not suggesting that parasitoids never regulate aphid colonies: top-down effects may still be important in regulating generalist aphid species that do not sequester plant secondary compounds. However, we found no evidence that cabbage aphid colonies are regulated by parasitoids or predators.

In these plant-aphid-natural enemy systems it is possible that the natural enemies are not greatly exposed to the bottom-up effects of plant defence chemicals on aphids (Ode 2006). However, the effects of secondary parasitism were not investigated in this study, secondary parasitoids can have a positive impact on herbivores by reducing the abundance of their natural enemies. Previous studies have shown that secondary parasitoids are also effected by plant phenotype and can have a significant impact on primary parasitoids (Harvey et al. 2003; Bukovinszky et al. 2008). Higher rates of secondary parasitoids may cause primary parasitoids to disperse to areas of with lower densities of secondary parasitoids, so it is possible that primary parasitoids have a greater effect on aphid colony growth rate very early in the season (Höller et al. 1993) which may not have been detected by our survey methodology.

THE IMPORTANCE OF WINGED APHIDS

Our results suggest that the production of winged aphids is not directly affected by plant glucosinolate phenotype. It shows a simple numerical relationship with aphid

colony size, which is affected by sinigrin presence. The production of winged aphids by colonies is of ecological importance for two reasons. First, winged dispersers form a generation of emigrants that may colonise other plants, including crops. Hence regulation of disperser production should scale up to regulation of aphid population number and sizes at larger scales. Second, parthenogenesis and aggregation by aphids can mean that a single monoclonal colony acts as a reproductive unit (Hodgson 2001). Given that a single aphid colony is unlikely to survive an entire season on a single foodplant, any selective pressures imposed by foodplant chemistry will be mediated by its effect on the production of dispersers (Hodgson 2001; Hodgson 2002). The correlation between colony size and plant phenotype means that the dispersal ability of the colony is correlated with sinigrin presence or absence, which may have an impact on the overall fitness of aphid clones.

The methods used in this study did not allow us to differentiate between winged aphids produced by the colony and those that joined the plant from surrounding colonies, nor did they allow us to account for aphids that leave the plant before being counted. Quantifying these effects in the wild is extremely difficult, but the large numbers of winged aphids observed were likely to have been dominated by those produced by the aphid colony being measured, and should correlate strongly with the actual total number of dispersers produced.

CONCLUSIONS

There appears to be no consensus on the relative importance of plant resistance (bottom-up mechanisms) and biological control (top-down mechanisms) on the regulation of herbivore populations (Hunter and Price 1992; Walker and Jones 2001; Ode 2006). Previous studies have presented evidence supporting bottom-up (Hawkins 1992; Cornelissen and Stiling 2006; Miller 2008), or top-down regulation (Kareiva and

Sahakian 1990; Costamagna and Landis 2006), or a combination of the two processes (Dyer et al. 2004; Stiling and Moon 2005). We manipulated natural enemies on aphid colonies to determine the relative strengths of glucosinolate-mediated top-down and bottom-up effects and found that colony size was correlated with sinigrin presence. Our results indicate that plant defence chemicals can reduce the intensity of aphid attack through bottom-up factors, whereas top-down effects appeared to be less important in the regulation of cabbage aphid populations. Furthermore, the heritable chemical structure of plant defence metabolites differed in their regulatory impacts on aphid colonies, suggesting that the population genetics of plant secondary metabolites could be an important force in shaping the structure of food webs that contain wild cabbage as a basal food species.

Acknowledgments

The authors thank J. Blount, A. Barlow and N. van Dam for providing advice on HLPC and techniques for purifying desulphoglucosinolates, W. J. Grecian for fieldwork assistance and the EXPECT research group for comments on the manuscript. P. Behrens gave permission for access to field sites. Funding for this project was provided by the European Social Fund.

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Chapter 5

Do aphids drive differential selection for glucosinolate profiles in wild cabbage populations?

Abstract

1. Genotype-by environment interactions may maintain variation in natural populations. This mechanism requires that plants varying in a heritable antiherbivore defence trait show differential fitness in different environmental situations, such as the presence or absence of herbivores.

2. The presence of mealy cabbage aphids (*Brevicoryne brassicae*) and their natural enemies was manipulated on wild cabbage (*Brassica oleracea*) plants in natural populations. Plants in these populations vary in the presence or absence of sinigrin gluosinolate, which has a negative impact on aphid abundance and disperser production in the field.

3. Despite the negative effect of sinigrin on aphids, plants lacking sinigrin appeared to be highly tolerant to aphid attack and showed a consistently greater seed set compared to plants producing sinigrin. It is therefore unclear how the production of sinigrin is maintained in these populations. The removal of aphid natural enemies had no impact on seed set, indicating that aphid natural enemies do not act as an extension of the plants defences.

4. The results provide evidence that glucosinolate profile and aphids have significant effects on the fitness of wild cabbage plants in the field. However there was no evidence of genotype-by-environment interactions in this compartment of the cabbage-herbivore-natural enemy tritrophic system.

Introduction

A wide range of natural plant populations show substantial diversity in herbivore resistance mechanisms, such as plant secondary metabolites, across a wide range of taxa including Brassicaceae (glucosinolates) (Mithen et al. 1995b; Moyes et al. 2000), Lamiaceae (iridoid glucosides) (Harvey et al. 2005), Fabaceae (cyanogenic glycosides) (Kakes and Chardonnens 2000; Richards and Fletcher 2002), and Apiaceae and Rutaceae (furanocoumarins) (Berenbaum et al. 1986). Understanding the mechanisms maintaining this diversity remains a focal topic in plant evolutionary biology (Baucom and Mauricio 2008).

A significant proportion of a plant's environment may consist of the influence of other species' phenotypes; i.e. 'extended phenotype' and 'community genetics' theories (Whitham et al. 2003; Bailey et al. 2006; Bangert et al. 2006; Tetard-Jones et al. 2007). Herbivores are an important aspect of a plant's environment and can be expected to exert substantial selective pressure, particularly on plant chemical defences. For example, a study by Berenbaum and Zangerl (1998) identified a correlation between the frequency of resistance trait (the production of furanocoumarins) in wild parsnip populations, and the furanocoumarin detoxification ability of the parsnip webworm: the primary herbivore species. A further indication that variation in the herbivore community can act as a mechanism promoting the maintenance of variation in wild populations is provided by evidence that a change in the phenotypes favoured by selection occurs after the re-association of a host plant with its specialist herbivore (Zangerl et al. 2008).

Interactions between genetically determined plant defence chemistry and variable environmental conditions, such as the presence or absence of an herbivore species, can result in four possible outcomes. First, plant fitness may not differ between genotypes or in different environments (Fig. 5.1a); in this scenario the observed genetic

variation in the population is due to genetic drift. Second, plant fitness might vary under different environmental conditions but the genotypes have a similar fitness within each environment (Fig. 5.1b): in this situation, genetic diversity is again maintained through drift due to a total lack of directional selection on plant genotypes within each environment. Third, genotypes might show variation in fitness but there is no effect of environment and the relative difference in fitness between the genotypes is identical across all environmental conditions (Fig. 5.1c). In this situation there is a consistent directional selection favouring a genotype and we would expect that this genotype would eventually spread to fixation within the population. Fourth, the relative fitness of individual genotypes might vary under different environmental conditions (Fig. 5.1d): i.e. genotype-by-environment interactions (Gillespie and Turelli 1989). Defence-chemical diversity in plant populations may be maintained if the production of a particular secondary metabolite gives a fitness advantage under certain conditions (i.e in the presence of an herbivore species), but has a negative effect on plant fitness in another (such as in the absence of herbivores or in the presence of a different species). This means that fluctuations in the presence of abundance of an herbivore could prevent a single genotype gaining a consistent advantage and will reduce the likelihood of alleles becoming fixed in the population, thus maintaining polygenic variation.

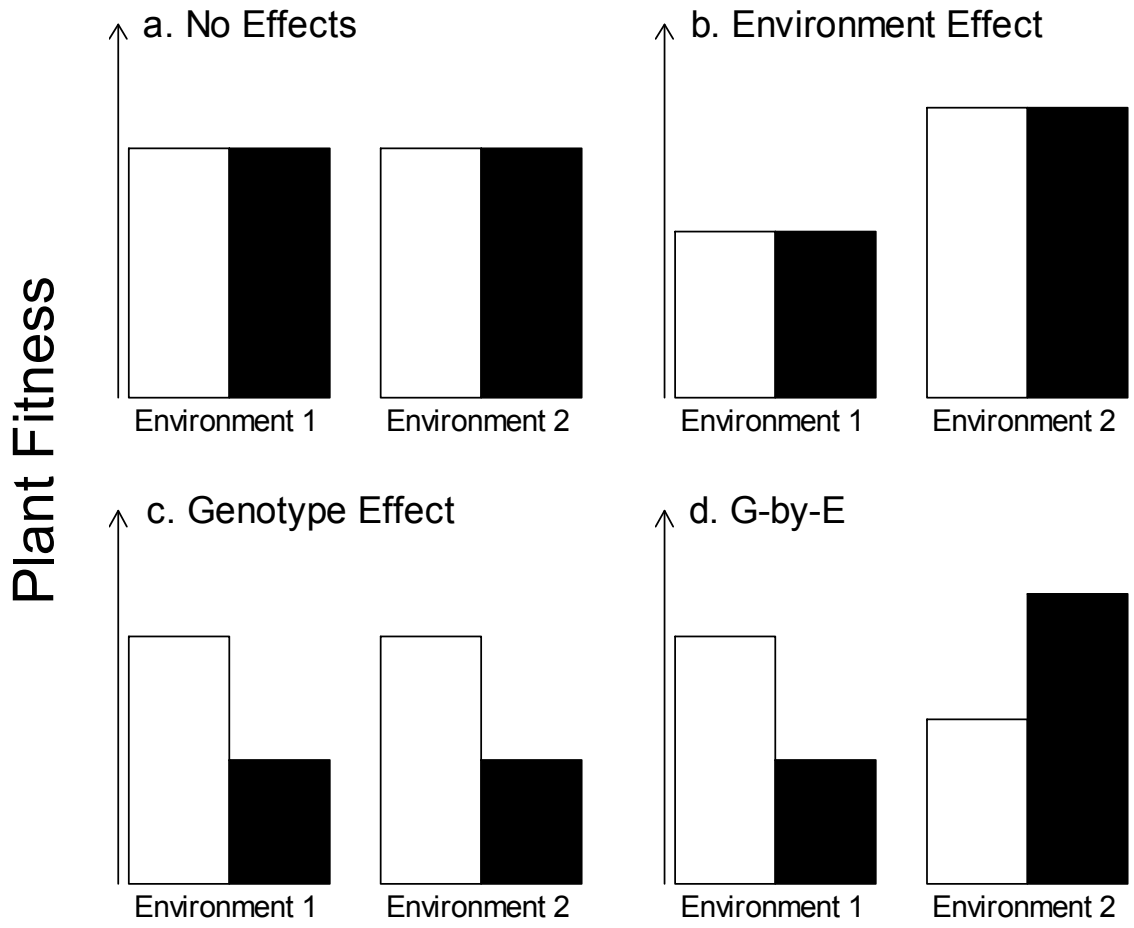

Figure 5.1. Graphical representation of the four possible outcomes on plant fitness of interactions between different plant genotypes (represented by black and white bars) and environment.

Demonstrating differential fitness of phenotypes in the presence or absence of herbivores is a fundamental requirement for herbivore-mediated, genotype-by-environment interactions to play a role in the maintenance of diversity. Wild cabbage (*Brassica oleracea* L. var. *oleracea*, subsequently referred to as *B. oleracea*) populations offer a good opportunity to test the impact of herbivore attack on the fitness of plants showing phenotypic variation in defence chemical profiles. Natural populations of wild cabbage show qualitative and quantitative variation in glucosinolates (Mithen et al. 1995b; Moyes et al. 2000), which are a class of secondary metabolites involved in herbivore defence (Li et al. 2000; Wittstock et al. 2004). The genetic architecture of glucosinolate production is well characterised and the production of aliphatic glucosinolates is known to be under strict genetic control by 5 loci (Magrath et al. 1994; Parkin et al. 1994; Mithen et al. 1995a; Gao et al. 2007), providing a strong link between the aliphatic glucosinolates produced by a plant and its genotype.

The focal herbivore in this study is the mealy cabbage aphid (*Brevicoryne brassicae*). Aphids are well known agricultural and garden pests: their parthenogenic reproduction results in heavy infestations within a short time and this can have severe impacts on plants (Dixon 1998; Goggin 2007). The cabbage aphid is a *Brassica* specialist and is stimulated to feed by the secondary metabolites (glucosinolates) (Wensler 1962; Nault and Styer 1972) involved in herbivore defence. The aphid even hijacks the plants own defence compounds by sequestering glucosinolates for use against its own predators and parasitoids (Bridges et al. 2001; Kazana et al. 2007; Pratt et al. 2008). *B. brassicae* can be found on wild cabbage during the flowering period (April-June) when heavy infestations can be seen covering the flowering stems. Furthermore, previous work indicates that cabbage aphids are less likely to be found on plants producing the glucosinolate sinigrin (Chapters 2 and 3) and the size of aphid colonies is negatively affected by sinigrin presence: this negative response of aphids

appears to be caused by a bottom-up effect of plant chemistry rather than a top-down effect of aphid natural enemies. But does the reduction in the probability and intensity of aphid infestation, in plants investing in sinigrin, translate to an increase in fitness? To investigate the effect of aphid attack on plants producing or lacking sinigrin we manipulated aphid colonies and their natural enemies on wild *B. oleracea* plants during the flowering season.

Based on previous results from work carried out in wild cabbage populations we can predict two outcomes that should be observed if genotype-by-environment interactions are involved in the maintenance of glucosinolate diversity:

1) Aphid attack is harmful to plants, but previous work indicates that sinigrin deters aphids. Therefore, in the presence of aphids, plants producing sinigrin will show a higher seed set compared to plants producing only butyl-derived glucosinolates.
2) The repulsion of aphids by sinigrin production may have an ecological cost. Therefore, in the absence of aphids, seed set in plants producing sinigrin should be lower relative to alternative glucosinolate phenotypes.

Materials and Methods

FIELD SITES

Flowering *B. oleracea* plants at three sites; PC1 (50°10'N, 5°42'W), PC2 (50°12'N, 5°42'W), and PC3 (50°10'N, 5°41'W) were used in the study. All three sites are located within a 5km stretch of coastline consisting of several sheltered coves on the south coast of Cornwall. There is little variation in aspect or altitude between sites. Plant gene flow between the sites is highly probable: the coastline is popular with walkers which could lead to long distance seed dispersal (Wichmann et al. 2009) and pollinator-mediated gene flow is likely as distance between the sites is less than the

potential range of many bee species (around 10km) (Visscher and Seeley 1982; Pasquet et al. 2008). A transect was laid at each site along which flowering plants were selected at random and marked using individually-numbered copper tags. The basal stem diameter was measured for each marked plant as a standard measure of plant size (e.g. Stokes et al. 2004), and therefore as a covarying predictor of seed set. The transect start and end points were recorded using a GPS receiver to enable the plants to be found during subsequent visits. The field experiment was carried out over two years during the flowering season in May-June 2007 and 2008.

GLUCOSINOLATE EXTRACTION AND ANALYSIS

Following Newton et al. (2009), leaf tissue was collected from each plant and glucosinolates were extracted, purified and separated by High-Performance Liquid Chromatography as described in Chapter 2. All plants studied produced the full range of butyl glucosinolate derivatives synthesised by gene products from the *GSL-elong, GSL-sulph*, *GSL-alk* and *GLS-oh* loci. The plants in this study exhibited one of two phenotypes, caused by the presence or absence of sinigrin, controlled by gene products from the *GSL-pro* locus. In this thesis we investigate the response of cabbage aphids to natural variation in the presence or absence of individual aliphatic glucosinolates, and not quantitative variation in glucosinolate concentrations. Indole glucosinolates are induced by herbivory (Bodnaryk 1992), therefore we focused our study on the presence/absence of heritable aliphatic glucosinolates. Although quantitative differences in glucosinolate concentration will influence aphid responses, the presence or absence of individual aliphatic glucosinolates is a well studied, heritable component of cabbage defence.

In April 2007, 30 plants producing sinigrin (2-propenyl glucosinolate) and 45 plants producing only butyl glucosinolate derivatives were used in the study and divided into two treatments: half of the plants were kept free of aphids for the flowering period (the 'no aphid' treatment) and *B. brassicae* colonies were initiated on half of the plants by adding 5 aphids to a developing flower stem (the 'control' treatment). If an aphid colony was already present on plants allocated to the control treatment, aphids were removed until 5 remained. The aphids were sourced from a pool collected from at least 10 randomly chosen colonies within the same cabbage population as the experimental plants. The plants were visited every three days throughout the flowering period to maintain the no aphid treatment by removing any aphids found on the plants with a fine paintbrush and record the numbers of unwinged, winged and parasitised aphid mummies, as well as all aphid natural enemies on plants in the control treatment.

In 2008, a total of 34 'sinigrin' plants and 63 'no sinigrin' plants were selected for the study. The 'no aphid' and 'control' treatments were applied as in 2007 and an additional 'aphid natural enemy removal' treatment was included in the experimental design. Aphid colonies were initiated on plants assigned to the control and natural enemy removal treatments as in 2007. In the control treatment all aphid natural enemies were allowed to attack the colonies, while in the natural enemy removal treatment all parasitised aphids and aphidophagous insects were removed from the colonies using fine forceps throughout the survey period. In order to standardise the initiation of aphid colonies, any aphids present were removed twice a week from all plants for three weeks prior to the start of the experiment in April 2008. From then on plants were visited every three days to maintain the treatments and record the numbers of unwinged, winged and parasitised aphids as well as numbers of aphid natural enemies on plants in the control treatment. At the end of the flowering period the total number of successful

and failed seed pods on each plant was counted: failed pods were identified by the presence of a withered flower stalk that had no developing pod. In August 2008 10 mature seed pods were collected from each plant and returned to the laboratory to be counted and weighed: this enabled the mean number of seeds per pod to be estimated for each plant and this figure could then be multiplied by the total number of successful pods to obtain an estimate of the total seed set.

STATISTICAL ANALYSIS

All statistical analysis was carried out using R 2.7.0. The effect of aphid presence, plant phenotype and year of experiment on seed set was analysed using a linear mixed effects model. Plant basal stem diameter was also included in the main effects to account for the effect of plant size on seed production. We accounted for spatial structuring of plants and repeated sampling of plants across years by modelling plant population and plant identity as random effects. Seed set was log-transformed to standardise and homogenise residuals.

A similar mixed modelling approach was used to investigate the effect of removal of aphid natural enemies on seed set in all plants studied in 2008. The effect of all treatments ("control", "no aphids" and "no aphid natural enemies") and plant phenotype on seed set was tested using a generalised linear mixed effect model with Gaussian errors and an identical random effects structure to that used in previous analyses. Seed set was log-transformed as before and plant basal stem diameter was also included as a covariate. We tested significance of explanatory variables and their interactions in all analyses using model simplification and likelihood ratio tests (Crawley 2007).

The effect of aphid colony size on seed set was investigated using a linear mixed effects model with plant population and plant identity included as random effects. The

cumulative number of aphids (i.e. the total number of unwinged aphids observed on each plant over the course of the study period) on a log scale was used as a measure of aphid colony size. Year of study, plant phenotype and basal stem diameter were also included as fixed factors and log transformed seed set was analysed as the response variable.

Prior to these analyses, we confirmed a lack of spatial structuring of phenotypes within each site using a Mantel test (Mantel 1967) to investigate spatial autocorrelation between sinigrin phenotype and distance along the transect, which might have resulted in confounding effects of environmental stresses on aphid colony dynamics and plant seed set.

Results

EFFECT OF APHID AND NATURAL ENEMY PRESENCE ON SEED SET

HPLC analyses confirmed that plant chemical phenotypes varied only in the presence or absence of sinigrin, i.e. all plants produced the full range of butyl-derived aliphatics. The sites showed slight differences in the frequencies of the two glucosinolate phenotypes (frequency of plants producing sinigrin in addition to all butyl-derived aliphatics: PC1 = 0.14, PC2 = 0.41, PC3 = 0.32). Results from the Mantel tests showed that there was no spatial autocorrelation in phenotype distributions along transects in each population (PC1 $P = 0.36$, PC2 $P = 0.57$, PC3 $P = 0.55$).

The interaction between aphid presence and year had a significant effect on seed set ($\chi^2_1 = 5.68$, $P = 0.02$): the presence of aphids had a negative effect on seed set in 2007 but this effect was lost in 2008 (Fig. 5.2). Plant phenotype also had a significant effect on seed production: plants lacking sinigrin produced a greater seed set ($\chi^2_1 = 9.78$, $P < 0.01$, Fig. 5.2). The negative effect of sinigrin on seed production was observed regardless of aphid presence or absence as there was no significant interaction between

plant phenotype and aphid treatment in their effect on seed set (χ^2_1 <0.01, P = 0.99). As expected, plants with a larger basal stem diameter had a greater seed set (χ^2_1 =24.52, P <0.01).

The removal of aphid natural enemies had no significant effect on seed set (χ^2_1 = 1.09, P = 0.58). There was no effect of an interaction between treatment and plant phenotype (χ^2_1 = 0.03, P = 0.98). This analysis of the 2008 experimental data confirmed the cross-year observation that plants producing only butyl-derived glucosinolates showed a greater seed set across all treatments (χ^2_1 = 11.08, P <0.01).

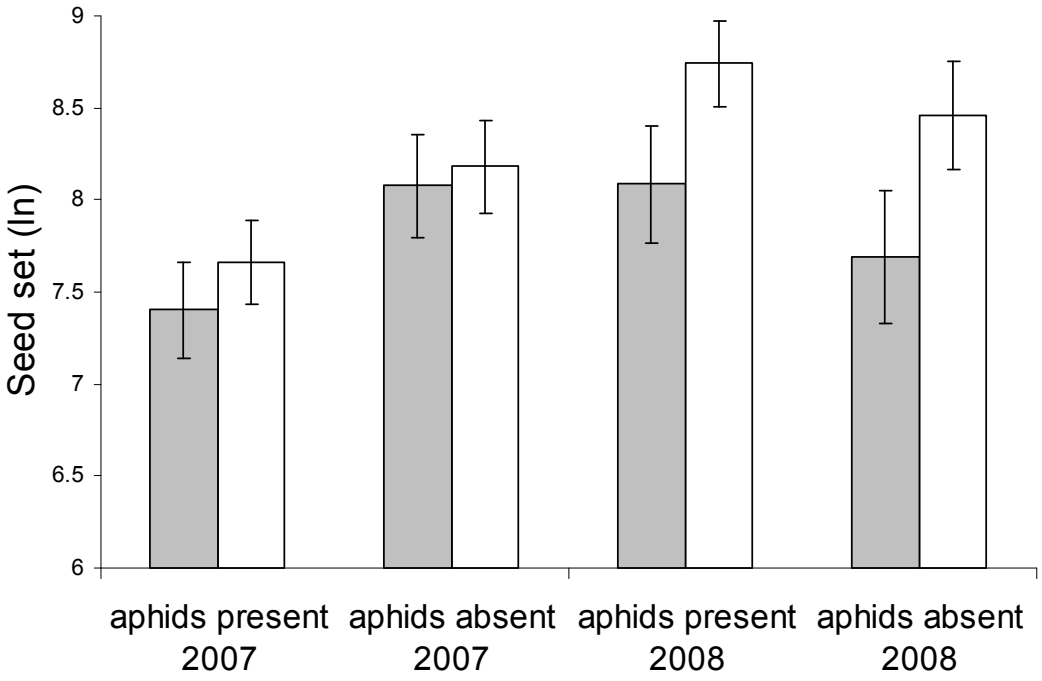

Figure 5.2. Mean seed set produced by plants in each treatment over the 2 years of the experiment. Plants lacking sinigrin are represented by white bars, plants producing sinigrin represented by the grey bars. Error bars show +/- 1 standard error. Means and standard errors calculated from estimates given by a linear mixed effects model for each year, ignoring the effect of basal stem diameter.

There was enormous variation in aphid colony sizes between plants which was not incorporated into the analysis above (previous models studied only the presence or absence of aphids). In addition, the mean cumulative number of aphids was substantially different between years (mean in 2007 = 2371, mean in 2008 = 640). Therefore, a more detailed analysis was carried out to investigate the impact of colony size on plant seed set and also to investigate whether the differing effect of aphid treatment in 2007 and 2008 was caused by variation in aphid numbers across the two study years. As there was no significant effect of the removal of aphid natural enemies the "control" and "natural enemy removal" treatments were analysed collectively.

There was no significant impact of the cumulative number of aphids on seed set in 2008 (t= 1.38, $P = 0.19$), however in 2007 the slope was negative (test of interaction between year and cumulative aphid numbers; $\chi^2_1 = 5.93$, $P = 0.01$, Fig 5.3). The effect of sinigrin production on seed set was negative ($\chi^2_1 = 9.44$, $P < 0.01$) and there was no significant interaction between plant phenotype and the cumulative number of unwinged aphids ($\chi^2_1 = 0.01$, $P = 0.93$) therefore, the negative effect of sinigrin was consistent across all colony sizes. The effect of basal stem diameter ($\chi^2_1 = 22.24$, $P < 0.01$) on seed set remained highly significant (Fig. 5.4).

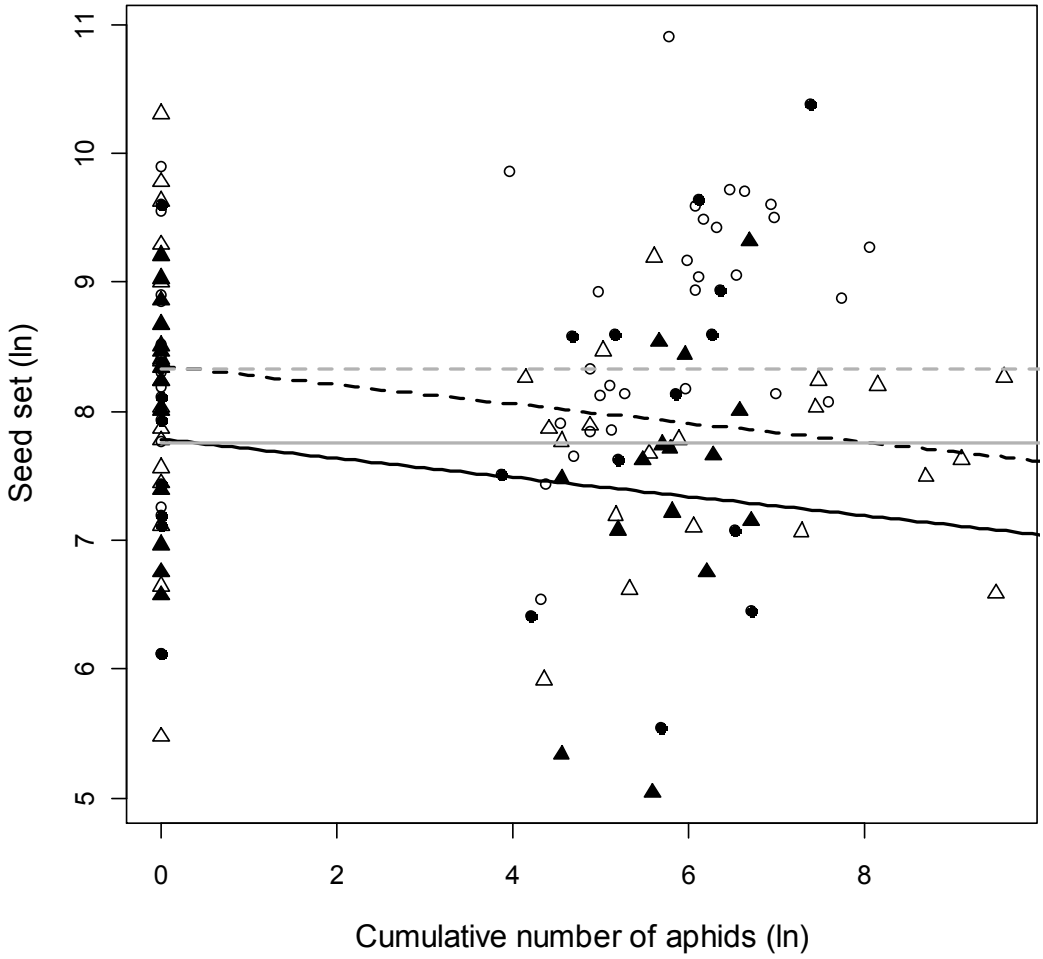

Figure 5.3. Plot of the effect of the cumulative number of aphids on plant seed set for 2007 (circles) and 2008 (triangles) for plants producing only butyl-derived glucosinolates (open symbols) and plants producing butyls and sinigrin (filled symbols). Fitted lines are based on estimates from the linear mixed effects model used for analysis and show the response for an average plant in an average population with a mean basal stem diameter for 2007 (black lines) and 2008 (grey lines). Dashed lines represent plants lacking sinigrin, solid lines represent plants producing sinigrin.

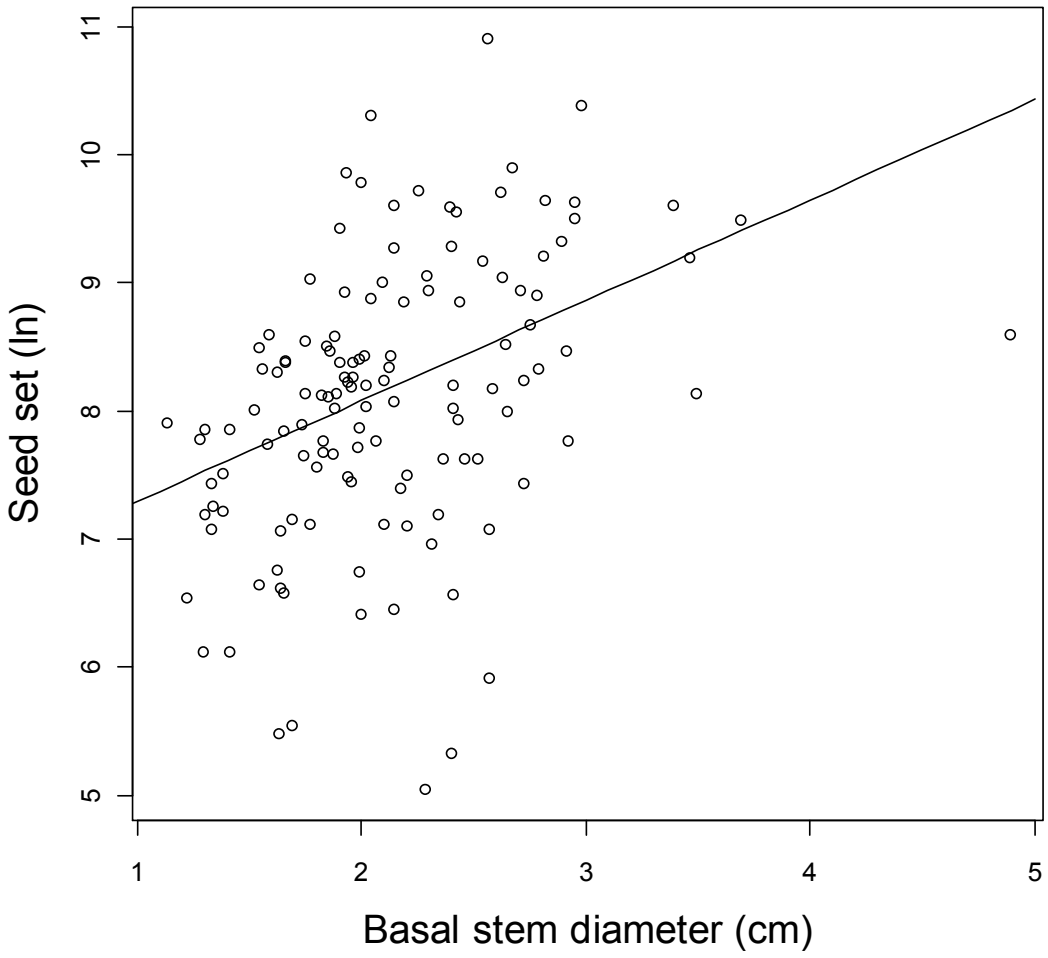

Figure 5.4. The relationship between basal stem diameter and seed set. Fitted line calculated from estimates given by a linear mixed effects model with treatment, phenotype, year and basal stem diameter as main effects and plant population as a random effect. The line shows the relationship between seed set and basal stem diameter for a plant with a mean colony size, for the average year and phenotype.

Discussion

EFFECT OF ENVIRONMENT AND PHENOTYPE BUT NO EVIDENCE FOR G-BY-E

One of the major drivers behind research assessing the fitness costs of resistance to herbivory is to develop an understanding of why resistance polymorphisms are maintained in populations (Purrington 2000). Previous work has shown that the phenotype favoured by selection can vary depending on the presence of an herbivore species (Zangerl and Berenbaum 1997). We find that there is a significant environment effect: plant seed set is greater in the absence of aphids. We also detected a significant effect of genetically determined variation in plant chemistry on seed set: plants producing only butyl-derived aliphatic glucosinolates have a higher seed set compared to plants producing butyls and sinigrin. However, this phenotype effect was observed in the presence and absence of aphids and therefore there is no evidence of a genotype by environment interaction.

EFFECT OF APHID ATTACK ON SEED SET

Wild cabbage plants producing sinigrin showed a lower seed set in the presence and absence of aphids compared to plants producing only butyl-derived glucosinolates. Seed set produced by plants lacking sinigrin also showed a negative response to aphid colony size, while plants producing only butyl-derived aliphatics showed no response to colony size. The results indicate that the production of sinigrin incurs a cost in terms of seed set across all levels of aphid attack. This study was carried out using the variation in plant phenotypes present in natural populations, however, this limited the number of plants of each phenotype that were available to be divided among treatments and restricted the sample size. Furthermore, although only aphids and their natural enemies were manipulated in this study, several other species feed on plant reproductive tissues and may have a confounding effect on the results: for example, seed weevils

(*Ceutorhynchus assimilis*) are attracted to plants with higher concentrations of aliphatic isothiocyanates (Cook et al. 2006) (breakdown products of glucosinolate hydrolysis) and can have a significant impact on *B. oleracea* seed set (Moyes and Raybould 2001). An additional disadvantage of studying wild plants was that we could not produce a full profile of each plant's primary and secondary metabolism. Therefore we can only implicate the effects of plant profile on seed production and interactions with aphids. However, inclusion of plant basal stem diameter in our analyses provided some control for correlations between plant phenotype and quality. The Mantel tests showed no spatial grouping of plants in terms of sinigrin phenotype, which also supports our interpretation that differences in seed production were due to plant chemical phenotypes rather than confounding environmental variables.

There are two distinct defence mechanisms that plants can employ against herbivore attack: the reduction of the effect of damage on plant fitness (tolerance), and the reduction in the intensity if herbivore attack (resistance) (Crawley 1983). The results suggest that wild cabbage plants lacking sinigrin are highly tolerant of aphid attack: although the presence of aphids had a negative effect on seed set in 2007 there was no evidence of a decline in seed set in the presence of aphids in 2008 and, in the presence of aphids, there was no decline in seed set with increasing colony size. While plants lacking sinigrin appear to show a tolerance defence mechanism, previous results providing evidence that aphid colony size is reduced on plants producing sinigrin (Ch4) indicates that this phenotype may be employing a resistance defence mechanism. However, although we detect a cost in terms of seed set for sinigrin production and find evidence that sinigrin leads to a reduced intensity of attack by aphids, there appears to be no fitness advantage (in terms of seed set) across the range of environmental conditions studied.

Previous work on the responses of mealy cabbage aphids to sinigrin glucosinolate in the field (Chapters 2 and 3), together with the observation that aphids are present in high abundance on reproductive plant structures during the flowering season, highlighted aphids as a candidate herbivore species that may influence the maintenance of heritable diversity in glucosinolates in wild cabbage populations through genotype-by-environment interactions. The findings do not support this theory, however, several steps in the methodology used for calculating plant seed set may have introduced sources of error into the estimate. In addition, seed set is an incomplete measure of plant fitness and aphids may have a greater impact on some other correlate of plant fitness: population models suggest, in perennial plants, variation in survival may be a more important measure of plant fitness (Stokes et al. 2004).

A question remains about how the observed variation in phenotypes is maintained despite evidence supporting consistent directional selection against plants producing sinigrin. It is possible that sinigrin confers an advantage in the presence of aphids in some other aspect of plant fitness that was not measured. Alternatively, as wild cabbage is attacked by a variety of herbivore species it is possible that if herbivores show differential responses to defence chemical phenotypes in the natural populations (Fritz et al. 1987; Maddox and Root 1990; Dungey et al. 2000; Bangert et al. 2006; Wimp et al. 2007); and see chapters 2 and 3), fluctuations in the herbivore community may lead to the maintenance of diversity in glucosinolate profiles.

EFFECT OF NATURAL ENEMIES ON SEED SET

Herbivore natural enemies have been suggested to have beneficial fitness effects on plants by acting as an extension of the plants' defences (Price et al. 1980). The theory of top-down regulation of herbivores is fuelled by studies demonstrating that natural enemies can distinguish between the volatile compounds produced by host-

infested, non-host infested and undamaged control plants plants (De Moraes et al. 1998; Du et al. 1998; Rose et al. 1998; van Poecke et al. 2003; Girling et al. 2006). In addition, several studies have demonstrated that the removal of herbivore natural enemies results in decreased plant productivity (Gomez and Zamora 1994; Stiling and Moon 2005; Tooker and Hanks 2006).

The results presented here show no effect of aphid natural enemy removal on seed set, which corresponds to the findings in Chapter 4 which showed that relieving aphid colonies from the pressure of natural enemies had no effect on aphid colony size. The natural enemy removal treatment was only applied in 2008, a year of lower aphid abundance compared to 2007. It is therefore possible that natural enemies play a greater role when aphids are more abundant. The majority of studies that demonstrate a positive effect of natural enemies are carried out in crop species grown in monoculture and where the density of pests is greater (reviewed in Hunter 2002): the searching efficiency of parasitoids has been shown to be higher in less complex plant communities (Gols et al. 2005). In addition, Karban (2007) has shown that even if damage results in an increase in the presence of herbivore predators and parasitoids around the plant, this does not necessarily result in a decrease in the level of herbivore attack in natural systems.

CONCLUSIONS.

The findings of this study do not support the proposal that, in the presence of aphids, the negative effect of sinigrin on aphid attack results in plants producing sinigrin to have a higher seed set relative to plants lacking sinigrin. In addition, the production of sinigrin appears to be costly in terms of seed set across all levels of aphid attack. The costs of chemical resistance have been attributed to mechanisms such as trade-offs between resistance and tolerance (Stowe 1998) and ecological costs such as the

attraction of pollinators (Strauss et al. 1999). We have not yet identified a mechanism for the cost of sinigrin production on seed set, relative to the production of butyl-derived glucosinolates.

In summary, plant seed set was affected by variation in the biotic environment and also by variation in genetically determined plant secondary metabolites. However there was no interaction between these variables on plant fitness: hence the results provide no evidence of genotype-by-environment interactions. Plants producing only butyl-derived glucosinolates appeared to be highly tolerant to aphid attack. Conversely, plants producing butyls and sinigrin incurred a cost in terms of seed set that was not alleviated under any of the environmental conditions studied.

Acknowledgements

I would like to thank J. Blount, A. Barlow and N. van Dam for providing advice on HLPC and techniques for purifying desulphoglucosinolates, W. J. Grecian for fieldwork assistance and R. Daines for help with seed counts. P. Behrens gave permission for access to field sites. Funding for this project was provided by the European Social Fund.

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Chapter 6

Does herbivore attack have a differential effect on the fitness of glucosinolate phenotypes?

Abstract

1. Variation in aliphatic glucosinolate phenotypes is maintained within and between populations of wild cabbage (*Brassica oleracea*). This diversity may be maintained by genotype-by-environment interactions if the relative fitness of different phenotypes varies under different environmental conditions, such as in the presence or absence of certain herbivore species.

2. Herbivore surveys were carried out on wild cabbage plants of known glucosinolate phenotype across twelve naturally established populations. This chapter investigates the links between glucosinolate phenotype and herbivore presence and their effect on plant seed set.

3. Across populations, glucosinolate phenotype and plant population had a significant effect on seed set. Although there was no consistent effect of herbivores across populations, the presence of more abundant herbivores (snails and the cabbage aphid *Brevicoryne brassicae*) had an impact on plant seed set, however, the effect was highly variable between plant populations and highlighted the importance of environmental effects on interactions between plant chemistry, plant fitness and herbivory.

4. In summary, there is evidence that glucosinolate phenotypes have an impact on plant fitness and may be under selection pressures exerted by the local herbivore community. However, variation in plant seed set cannot be fully explained by genotype-by environment interactions between herbivore species presence and heritable variation in plant chemistry.

Introduction

A major reason for investigations into the impacts of herbivore resistance mechanisms on plant fitness has been to understand why resistance polymorphisms exist in natural populations (Purrington 2000). Populations of wild cabbage (*Brassica oleracea* var. *oleracea*) show significant, heritable intra- and inter-population variation in aliphatic glucosinolates: secondary metabolites involved in herbivore defence (Mithen et al. 1995; Moyes et al. 2000). The response of herbivores to qualitative variation in aliphatic glucosinolates varies between species, indicating that heritable variation in plant chemical profile can structure the natural herbivore community (Chapter 2 and 3). This leads to the hypothesis that glucosinolate polymorphisms may be maintained by top-down differential selection.

To support the theory that differential selection by herbivores could play a role in the maintenance of aliphatic glucosinolate diversity, genetically controlled variation in plant chemistry must be shown to affect plant fitness. Also of interest is how herbivore attack influences plants fitness under natural environmental conditions. Furthermore, if herbivore attack has a differential effect on the fitness of different glucosinolate phenotypes, genotype-by-environment interactions (Gillespie and Turelli 1989) could play a role in the maintenance of plant defence chemical diversity in natural populations.

Several studies provide evidence that glucosinolates affect plant fitness: total glucosinolate concentration within a plant has a negative effect on seed (Stowe 1998) and fruit (Mauricio 1998) production. Costs of the induction of the glucosinolate-myrosinase system have also been investigated, for example, Agrawal (2000) found that although induced plant defences were effective against herbivores there was no cost in terms of reduction in root and shoot biomass. However in a different *Brassica* plant

species, Agrawal et al (1999) found that the expression of inducible defences resulted in costs associated with pollen production.

In addition, there is evidence for ecological costs (which require interactions with other species (Strauss et al. 2002)) of glucosinolate production, such as reduction in pollinator service to plants with higher levels of glucosinolates (Strauss et al. 1999), or increased susceptibility to alternative herbivore species (Agrawal and Sherriffs 2001). These studies all contribute important knowledge on how the variation in the levels of expression or induction of plant resistance traits may be maintained. However, an alternative and seldom studied aspect of this topic is the costs that are associated with the production of structurally different glucosinolates. Furthermore, few studies have investigated the costs of resistance to herbivory across several natural plant populations (Bergelson and Purrington 1996; Strauss et al. 2002).

We surveyed 12 wild cabbage populations to investigate:

1. The effect of phenotypic variation in glucosinolates on plant seed set in wild cabbage populations
2. The effect of herbivore attack on seed production in natural plant populations
3. The relative fitness of glucosinolate phenotypes on seed set in the presence and absence of herbivores, i.e. is there evidence for genotype-by-environment interactions between plant chemistry and herbivory?

Materials and Methods

The methods used for herbivore surveys and determining the aliphatic glucosinolate profiles of wild cabbage plants are described in Chapter 2. In 2007 and 2008 additional plants were selected and marked, using the same selection criteria as in 2006, to replace plants that had died during the previous year in order to maintain approximately 40-50 survey plants in each population.

EFFECTS OF HERBIVORES AND GLUCOSINOLATE ON SEED SET

All marked plants that were flowering in 2008 were used to investigate the response of plant seed set to variation in glucosinolate profile and herbivore presence. In June 2008 an estimate of the number of seed pods produced by each plant was obtained by multiplying the number of pods in 1 metre of flowering stem with total flowering stem length. In September 2008, approximately 5-10 mature seed pods were collected from each plant and returned to the laboratory. The mean number of seeds per pod was calculated by dividing the number of seeds by the number of pods collected. Total seed set for each flowering plant was estimated by multiplying the number of successfully developed pods by the mean number of seeds per pod.

STATISTICAL ANALYSIS

All data analysis was carried out using mixed effects modelling in R version 2.7.0, with models simplified using likelihood ratio tests of significance. Standard model checks were used to verify normality and homogeneity of standardised residuals (Crawley 2007).

A linear mixed effects model was used to analyse the effect on plant seed set of glucosinolate profile and herbivore species presence on the plant in the year prior to seed production (determined from surveys in September 2007 and June 2008). Plant basal stem diameter was included as a covariate. Seed set was log-transformed to standardise and homogenise residuals. Plant population, nested within county were included as random effects. As above, the herbivores analysed were *P. rapae* (small white butterfly)*, P. brassicae* (large white)*, A. proletella* (whitefly), *B. brassicae* (mealy cabbage aphid), and snails (all snail species were analysed collectively).

The effects of interactions between plant population, glucosinolate profile, and herbivore presence on plant seed set was analysed using a generalised linear model with

a Gaussian error structure. As in the previous analysis, seed set was log-transformed and plant basal stem diameter was included as a covariate. The purpose of this analysis was to investigate the direction or the slope of any correlation between plant seed set and the effects of herbivores and glucosinolates within each population. This analysis was carried out on two herbivore species: *B. brassicae* and snails. These species were present in high enough abundance to show variance in presence/absence on flowering plants differing in glucosinolate profile within populations.

Results

EFFECTS OF HERBIVORES AND GLUCOSINOLATE ON SEED SET

The interaction between sinigrin and progoitrin had a significant effect on seed set ($\chi^2_1 = 3.97$, $P = 0.04$, Fig. 6.1): both glucosinolates had a positive effect on seed set but the interaction was negative, to the extent that while the production of either sinigrin or progoitrin resulted in an increase in seed set compared to plants lacking both glucosinolates, the production of both sinigrin and progoitrin lowered the positive effects on each glucosinolate on seed set. Seed set also showed a highly significant, positive correlation with plant basal stem diameter ($\chi^2_1 = 47.04$, $P < 0.01$, Fig. 6.2).

There was no correlation between seed set and any herbivore species interacting with sinigrin (*B. brassicae,* $\chi^2_1 = 0.90$, $P = 0.34$; *P. rapae*, $\chi^2_1 = 0.26$, $P = 0.61$; *P. brassicae*, $\chi^2_1 = 0.86$, $P = 0.35$; *A. proletella*, $\chi^2_1 = 0.21$, $P = 0.64$; snails, $\chi^2_1 = 0.01$, $P = 0.93$) or progoitrin (*B. brassicae,* $\chi^2_1 = 0.56$, $P = 0.45$; *P. rapae*, $\chi^2_1 = 0.12$, $P = 0.73$; *P. brassicae*, $\chi^2_1 = 0.76$, $P = 0.38$; *A. proletella*, $\chi^2_1 = 0.05$, $P = 0.83$; snails, $\chi^2_1 = 0.92$, $P = 0.34$). Nor was there any main effect of herbivores on seed set across populations (*B. brassicae,* $\chi^2_1 = 0.06$, $P = 0.81$; *P. rapae*, $\chi^2_1 < 0.01$, $P = 0.98$; *P. brassicae*, $\chi^2_1 = 0.05$, $P = 0.83$; *A. proletella*, $\chi^2_1 = 2.83$, $P = 0.09$; snails, $\chi^2_1 = 0.02$, $P = 0.90$).

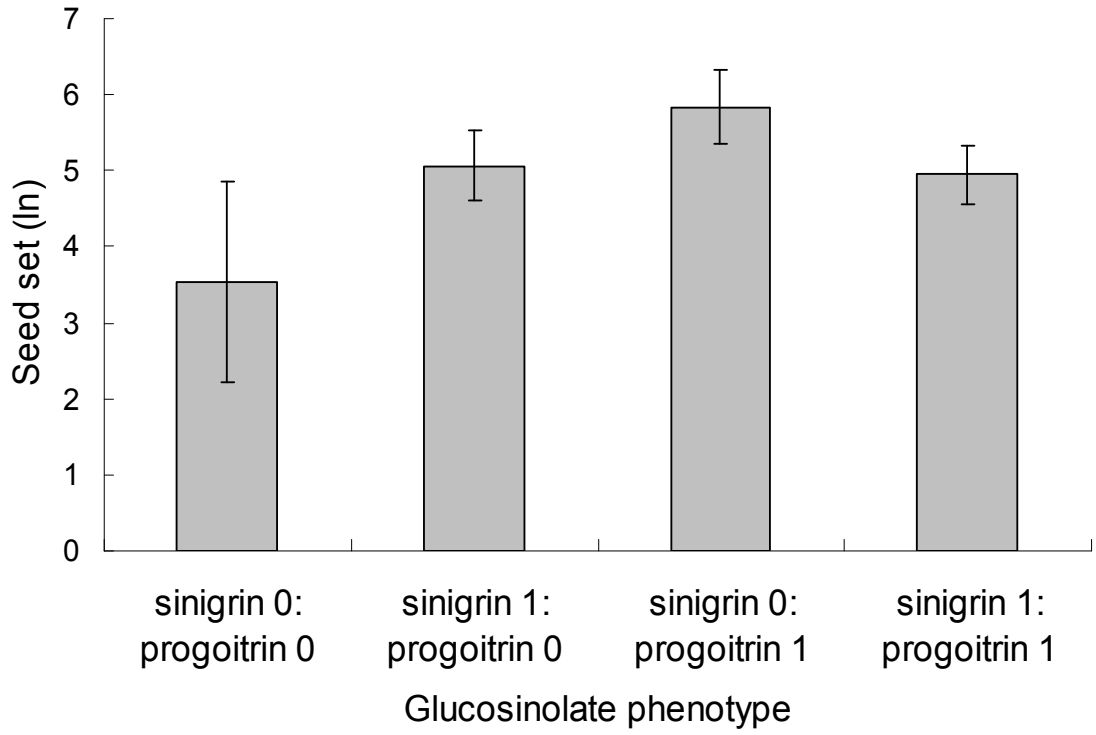

Figure 6.1. Mean seed set produced by plants differing in aliphatic glucosinolate profile. Only 2 plants lacked sinigrin and progoitrin. Means and standard errors were calculated from a linear mixed effects model of the effect of sinigrin and progoitrin on seed set, with county and plant population as random effects. Basal stem diameter was also included in the model in order to show the effects of sinigrin and progoitrin on seed set for a standardised plant size.

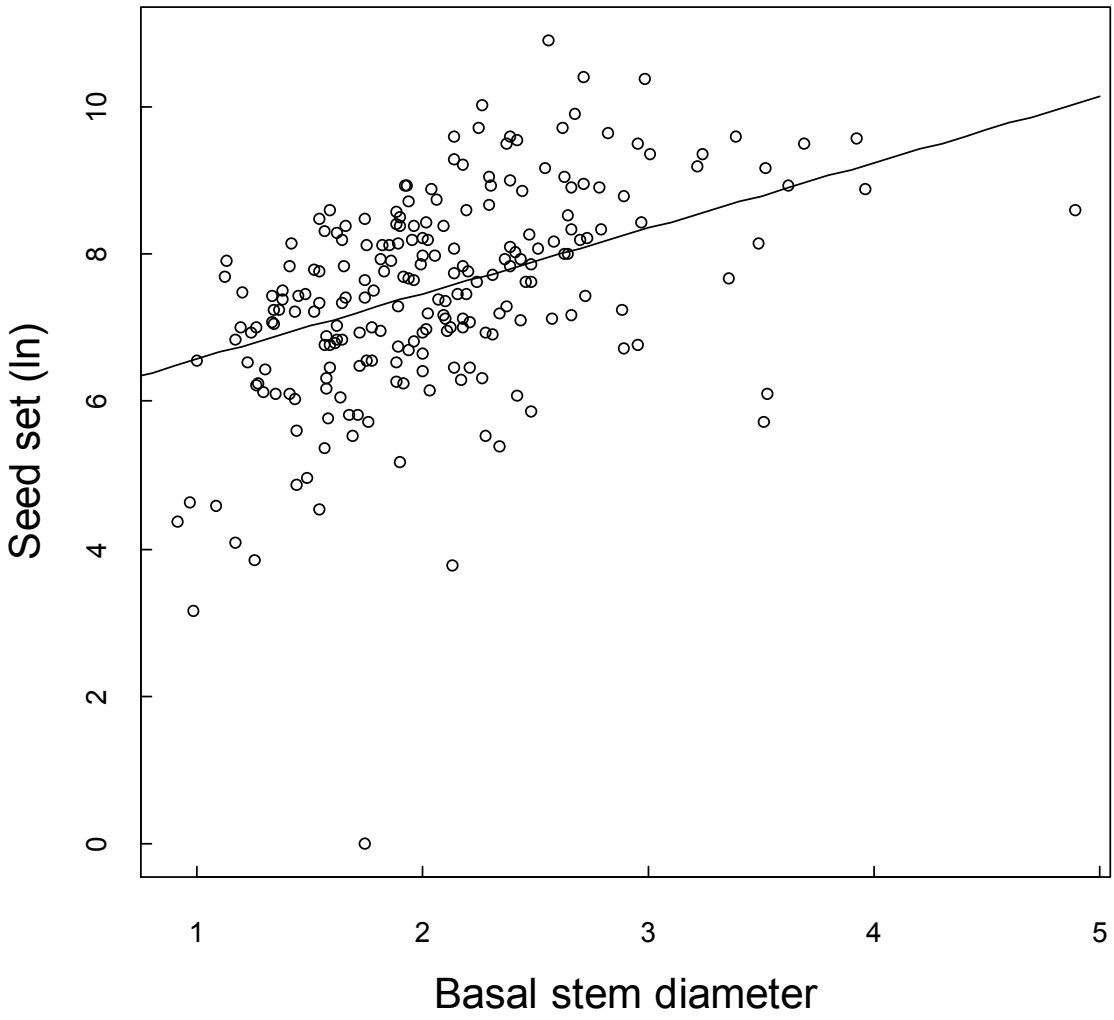

Figure 6.2. The correlation between plant basal stem diameter and seed set in 2008. Fitted line is based on a linear mixed effects model of the response of seed set to basal stem diameter with plant population and county as random effects.

Although there was no consistent effect of any herbivore species on seed set identified across all the plant populations, there were significant effects of glucosinolates and herbivore presence on seed set that varied between populations. Plant seed set is correlated with *B. brassicae* presence and sinigrin presence, and the direction of these correlations vary between populations (3-way interaction between aphid presence, sinigrin and population, χ^2_1 = 6.20, *P* =0.01, Fig. 6.3). Some populations, such as PC2, show a negative effect of *B.* brassicae presence regardless of phenotype (Fig. 6.3). Other populations (e.g. K2) show a positive correlation between herbivore presence and seed set, while some populations show no differences in seed set (e.g. WH and OH) (Fig. 6.3). There was no evidence of the interaction between *B. brassicae* presence and progoitrin on seed set (χ^2_1 = 0.11, *P* =0.74) but progoitrin presence did have a significant negative effect on seed production across all populations (χ^2_1 = 4.10, *P* =0.04, Fig. 6.4). This seems to contradict the findings of the previous analysis that found a positive response of plant seed set to progoitrin but a negative interaction between sinigrin and progoitrin across all populations. However, once the variation in seed set that is correlated with interactions between sinigrin, herbivore presence and population is absorbed, a significant negative effect of progoitrin is also observed. As in the previous analysis seed set was positively correlated with plant basal stem diameter (χ^2_1 = 19.40, *P* <0.01).

Similar results were found for the effects of snail presence, glucosinolates and population: seed set correlated with snail presence and sinigrin glucosinolates, but the effects varied with plant population (3-way interaction between snail presence, sinigrin and population, χ^2_1 = 9.96, *P* <0.01, Fig. 6.5). Once again, progoitrin had a significant negative effect (χ^2_1 = 5.10, *P* = 0.02) that showed no interaction with herbivore presence or plant population (χ^2_1 = 1.23, *P* = 0.27, Fig. 6.6), and basal stem diameter had a significant positive effect on seed set (χ^2_1 = 22.90, *P* <0.01).

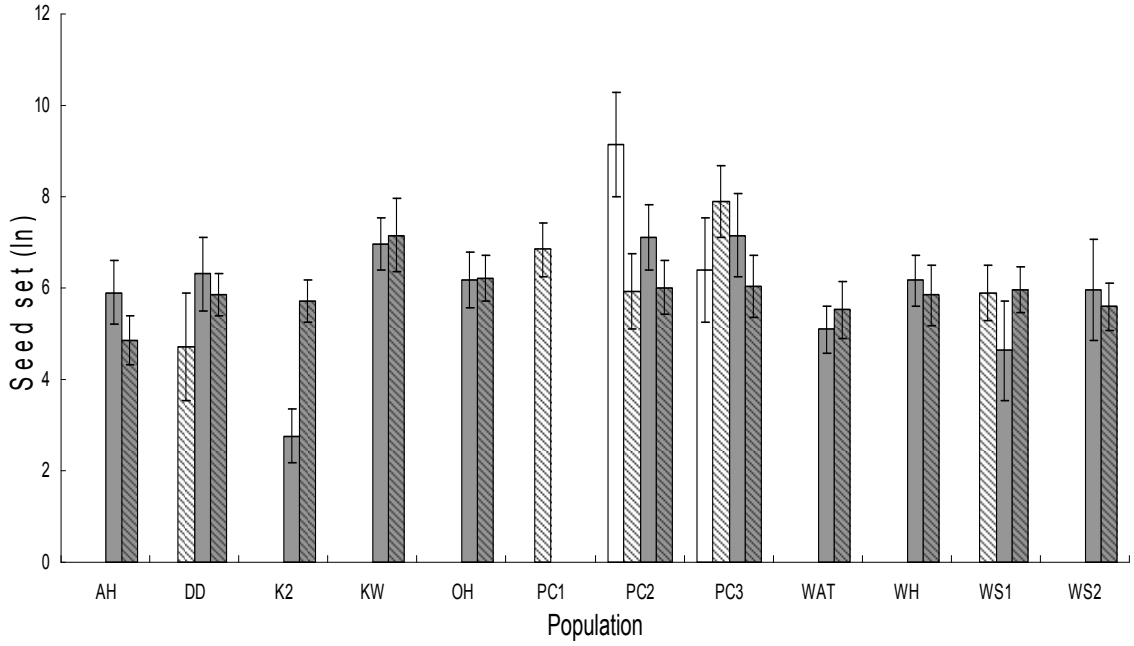

Figure 6.3. Seed set produced by plants lacking (white bars) and producing (grey bars) sinigrin in the presence (hatched bars) or absence (blank bars) of *B. brassicae* for each of the 12 populations studied. The numbers of bars per population vary as not all sinigrin and *B. brassicae* combinations were present in every population. Mean and standard errors calculated from a generalised linear model of the response of seed set to sinigrin and herbivore presence. Basal stem diameter was also included in the model as a covariate in order to show the effects of sinigrin and progoitrin on seed set for a standardised plant size.

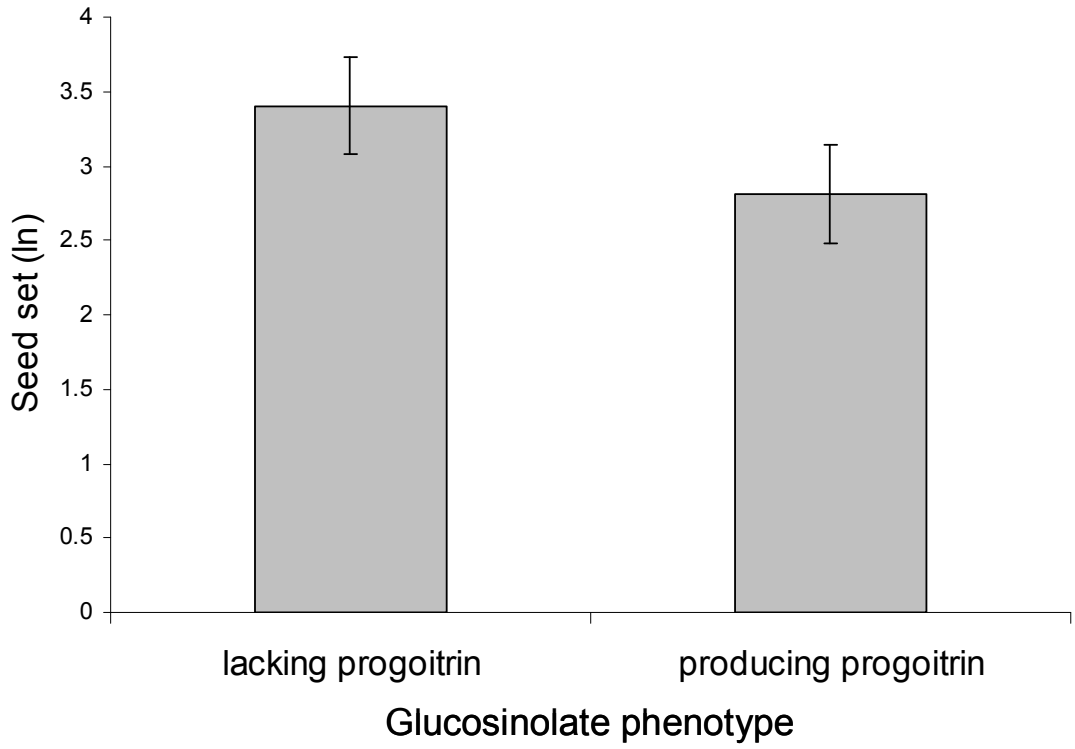

Figure 6.4. The effect of progoitrin on seed production. Means and standard error of the difference between the estimates calculated using a generalised linear model of the response of seed set to sinigrin and herbivore presence and plant population. Basal stem diameter was also included in the model as a covariate in order to show the effects of sinigrin and progoitrin on seed set for a standardised plant size. The results shown are the response of seed set in plant population AH, for a standardised basal stem diameter of 0cm, in the absence of sinigrin and in the absence of *B. brassicae*.

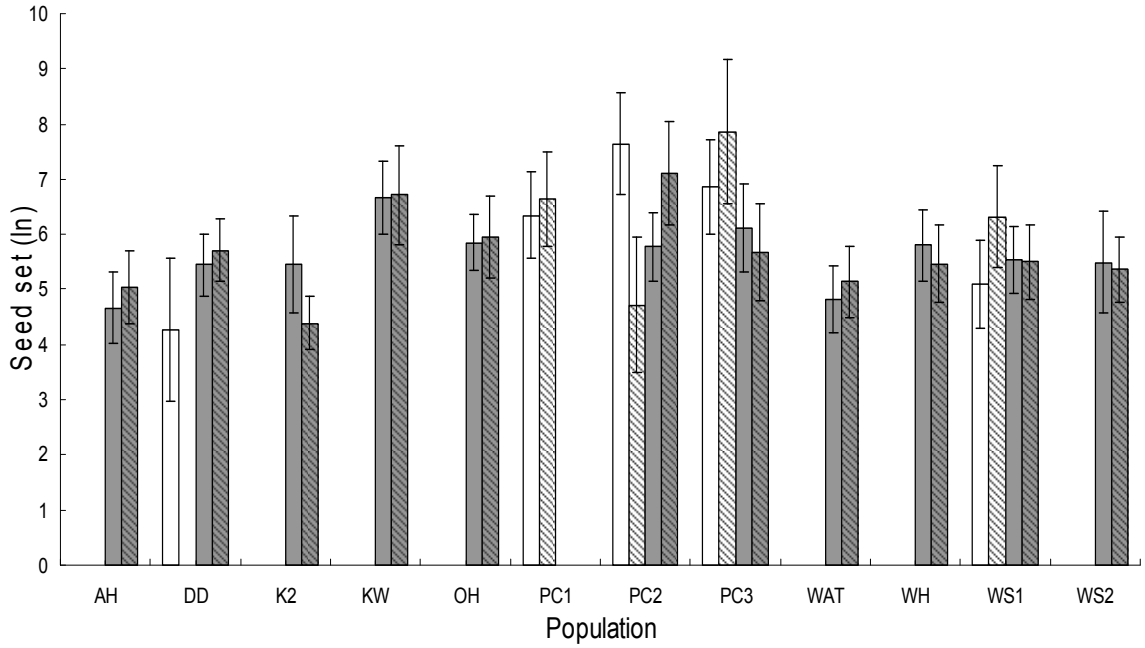

Figure 6.5. Seed set produced by plants lacking (white bars) and producing (grey bars) sinigrin in the presence (hatched bars) or absence (blank bars) of snails for each of the 12 populations studied. The numbers of bars per population vary as not all sinigrin and snail combinations were present in every population. Mean and standard errors calculated from a generalised linear model of the response of seed set to sinigrin and herbivore presence. Basal stem diameter was also included in the model as a covariate in order to show the effects of sinigrin and progoitrin on seed set for a standardised plant size.

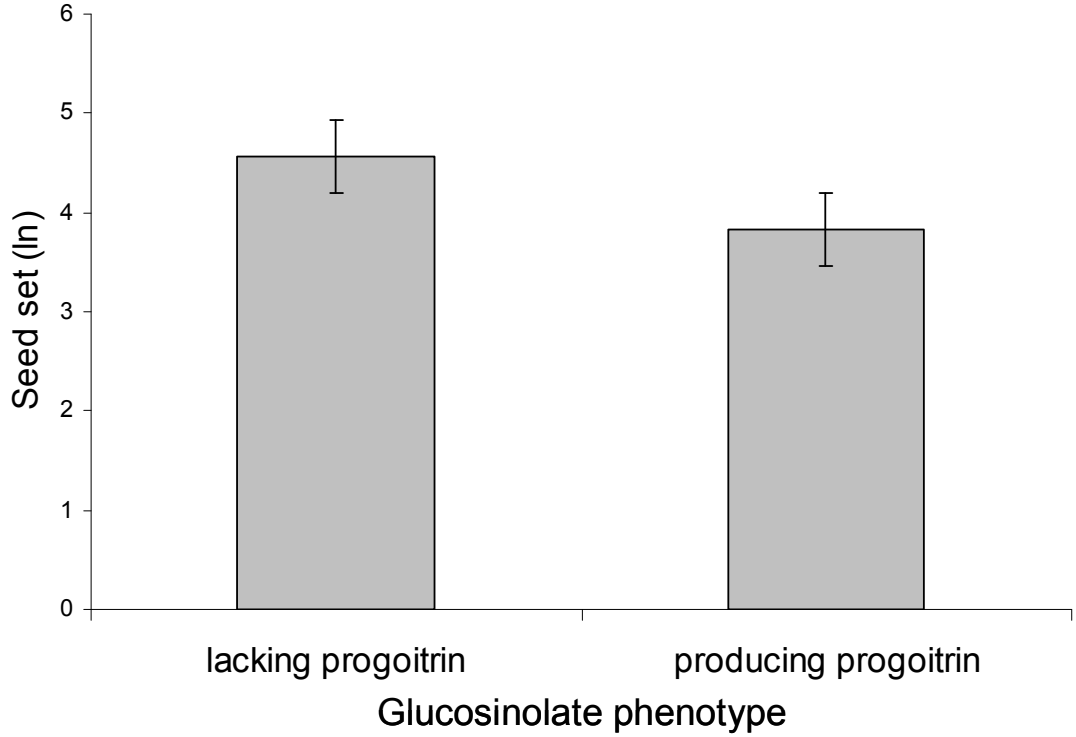

Figure 6.6. The effect of progoitrin on seed production. Means and standard error of the difference between the estimates calculated using a generalised linear model of the response of seed set to sinigrin and herbivore presence and plant population. Basal stem diameter was also included in the model as a covariate in order to show the effects of sinigrin and progoitrin on seed set for a standardised plant size. The results shown are the response of seed set in plant population AH, for a standardised basal stem diameter of 0cm, in the absence of sinigrin and in the absence of snails.

Discussion

Overall, there was a significant impact of glucosinolate profile on plant seed set, indicating that selection pressures can act on glucosinolate phenotypes and maintain plant chemical diversity in natural populations. There was also a signal of the effect of herbivore attack on seed production that varied between populations, however there was no general trend detected across all of the study populations. The results imply that although seed set is affected by heritable chemical traits and herbivore presence, there is no clear signal of genotype-by environment interactions on seed production by the plants.

GLUCOSINOLATES AND PLANT FITNESS

Seed set in wild cabbage plants was affected by the presence of sinigrin and progoitrin: each glucosinolate had a positive response, but the presence of both glucosinolates within a plant was correlated with a reduction in seed set. However, due to the fact that only two plants in the survey were found that lacked both sinigrin and progoitrin it is probable that the small sample size may have a disproportional effect on the results. If these plants were excluded from the analysis, figure 6.1 suggests that sinigrin may be the only glucosinolate influencing seed set.

EFFECT OF HERBIVORES ON THE FITNESS OF DIFFERENT GLUCOSINOLATE PHENOTYPES

There was no general trend for the effect of any herbivore species on plant seed set observed across populations. However, when the effect of herbivores was investigated at a finer scale, and the impacts of herbivore presence and glucosinolate profile were allowed to vary in magnitude and direction within each population, the different populations were found to show all possible combinations of results, i.e. positive, negative, and null effects of glucosinolates and positive, negative, and null

effects of snails and *B. brassicae*. The results indicate that environmental effects are important in moderating the cost of plant chemistry and herbivores on seed set. This analysis also showed a consistent, negative effect of progoitrin on plant seed set which seems to contradict the result obtained for the effect of this glucosinolate across populations. These results show that when the variation in the effects of herbivores and sinigrin is absorbed, progoitrin also has a negative impact on seed set over and above these effects.

EFFECT OF ENVIRONMENT AND PHENOTYPE BUT NO EVIDENCE FOR G-BY-E

The results of this study correlate well with the results presented in chapter 5, which showed that although both plant phenotype and aphid attack had an impact on seed set, sinigrin was costly across all levels of herbivore attack and so there was no evidence of genotype-by-environment interactions. A study by Zangerl and Berenbaum (1997) detected genotype-by-environment interactions in populations of wild parsnip (*Pastinaca sativa*): higher investment in furanocoumarin defence compounds resulted in a selective disadvantage in the absence parsnip webworms (*Depressaria pastinacella*). However, the parsnip webworm-wild parsnip populations may represent a closely coevolved ecological system: the herbivore feeds exclusively on plant species that produce furanocoumarins, and the plant is attacked by very few other herbivore species. The complexity of the herbivore community based around wild cabbage populations may restrict the ability to detect a clear signal with the survey methodology used in this study. Furthermore, there are many other aspects of glucosinolate defence chemistry that were not measured (such as induction of the glucosinolate-myrosinase system) which may also have an impact on seed production.

The measure of herbivory pressure on plants in the 12 months prior to flowering and seed production was determined by the presence of herbivores during only two

surveys. This survey technique provides a "snapshot" of the herbivore community and can only be an estimate of herbivory pressure experienced by plants differing in glucosinolate phenotype. Glucosinolates can decrease pollinator service to the plants (Strauss et al. 1999) and increase the damage cause by some specialist herbivores (Li et al. 2000; Lankau 2007), but can also decrease the damage caused by generalists (Lankau 2007). Thus, there is scope for interactions involving multiple herbivore species, which may mask the effects of individual herbivore species on plant seed set. Furthermore, the effect of natural levels of herbivore attack on plant seed set was investigated, and so herbivore presence may not be independent of plant chemical profile. Disentangling these interactions is difficult from the results gathered given the small number of field surveys and would require more intensive field and common garden studies.

DETECTING COSTS IN NATURAL POULATIONS

The costs of qualitative variation in plant chemistry are unlikely to be the same types of costs caused by quantitative variation. If plants have access to limited resources that must be allocated between growth, reproduction and defence (Bergelson and Purrington 1996; Strauss et al. 2002), then quantitative variation in herbivore defence compounds may impose allocation costs to fitness: for example Berenbaum et al (1986) found a negative correlation between the concentrations of different furanocoumarins produced by wild parsnip plants. Whereas ecological costs, mediated via interactions with another species (Strauss et al. 2002), are more probable as a mechanism for costs involving qualitative variation in herbivore resistance traits as evidence presented in previous chapters indicates that glucosinolates can mediate interactions between plants and herbivores. Herbivores vary in their responses to plant glucosinolate profile, therefore, the costs of producing different glucosinolates may vary depending on the

species of herbivores present. No attempt was made to define a mechanism for the costs of glucosinolate profile and herbivory in this study, so it is impossible to tell if the negative effect of sinigrin and progoitrin on seed was due to a lack of resources, or an ecological cost, such as a reduction in pollinator visitation rate (Strauss et al. 1999).

The costs of plant resistance to herbivores are more likely to be determined when the genetic background of the plants is genetically controlled, minimising the potential for genes that are correlated with the resistant trait to confound the effects of plant chemistry on fitness costs (Bergelson and Purrington 1996). These confounding effects cannot be resolved by this study, which presents correlations between plant fitness and qualitative variation in plant chemical phenotype in naturally established populations. However, patterns of resistance costs determined by studying the effects of herbivores and glucosinolates on seed set over a large number of natural populations across a wide geographical scale minimises the likelihood of spurious linkages between traits (Strauss et al. 2002).

CONCLUSIONS

Despite the caveats that arise from the limitations of determining the costs to plants of herbivore resistance traits in naturally established populations, we still find a signal for a general trend in the cost of variation in glucosinolate profile on seed across the 12 populations. Furthermore, in the more abundant herbivore species, there is also a signal that the fitness of phenotypes varies according to herbivore presence. The effect of herbivores on glucosinolate phenotype differed between plant populations, indicating the importance of environmental variation on these interactions. Consequently, although the variation on seed set was not fully explained by variation in glucosinolate profile and herbivore attack, there is some signal that variation in the local herbivore community can have an impact on the aliphatic glucosinolate phenotypes favoured by

selection, and provides support for differential selection by herbivores as a potential mechanism for the maintenance of intra- and inter-population diversity in genetically determined herbivore resistance traits.

Acknowledgements

Thanks to J. Blount, A. Barlow and N. van Dam for providing advice on HLPC and techniques for purifying desulphoglucosinolates, Matthias Wichmann for advice about the Dorset sites, Isobel Giblin and James Grecian for fieldwork assistance and Rosie Daines for help with seed counts. P. Behrens, T. Jones and Torbay Coast and Country Side Trust allowed access to field sites.

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Chapter 7

Discussion

The main theme of this thesis was to investigate the potential for natural variation in aliphatic glucosinolate profile to be maintained through selection pressures exerted by herbivores. There are four criteria that must be demonstrated, in order to support the hypothesis that differential selection by herbivores maintains variation in herbivore resistance traits in natural plant populations:

1. Natural populations must show significant, heritable variation in the resistance trait for selection to act on.
2. At least one species of herbivore should be shown to detect and respond to variation in secondary metabolite profiles in the wild.
3. The intensity of herbivore attack should fluctuate in order for the selection pressures exerted by the herbivore (or, if more than one species can detect and respond to the plant chemical variation, the herbivore community) to vary over time.
4. Herbivores and plant chemical variation affect plant fitness, and the relative fitness of chemical phenotypes varies in different environments, such as in the presence or absence of an herbivore species.

The first step in this investigation was to determine the magnitude of variation in glucosinolate phenotypes across a large number of natural populations. All of the phenotypic variation in the presence or absence of aliphatic glucosinolates in the 12 populations studied was due to allelic variation at two loci (Chapter 2): *GSL-pro*, which causes the formation of propyl glucosinolate side-chains; and *GSL-oh*, which causes the hydroxylation of butyl-derived glucosinolate side-chains (Magrath et al. 1994; Parkin et al. 1994; Mithen et al. 1995a).

Once the variation in glucosinolate profiles had been investigated and plants of known phenotype were marked in the populations, a three year herbivore survey was carried out. This revealed that the majority of herbivore species showed differential responses to the presence or absence of aliphatic glucosinolates (Chapters 2 and 3). Furthermore, the effects of glucosinolate variation on herbivores were detected at two ecological scales, within and between populations. Responses of herbivores within populations are expected if herbivores can detect phenotypic variation in glucosinolates, and are sufficiently mobile to be able to leave non-preferred plant phenotypes and move to alternatives. Inter-population scale responses of herbivores will occur when herbivores distribute themselves according to population scale patterns of secondary metabolites. Of the herbivores investigated in this thesis, it was the species that have the most restricted or passive dispersal mechanisms (snails, *A. proletella* and *B. brassicae*) that showed the most consistent responses over the three year survey period to glucosinolates at both ecological scales (Chapters 2 and 3). These herbivores were also the most abundant so it is likely that the result is partly due to sampling effort: the responses shown by the more abundant herbivores are more likely to be detected in a limited number of surveys per year, whereas a signal for the responses of the less abundant herbivore species may only be detected across all scales when a more intensive survey methodology is used. However, despite the limitations of the survey methodology, we still find that all herbivore species (with the exception of *Mamestra brassciae* which was only found on plants in the June survey of 2006) show a significant response to at least one glucosinolate, at one or more ecological scale.

The population-wide survey results demonstrate that intra- and inter-population variation in glucosinolates can structure the herbivore community at both ecological scales. However, more intensive studies carried out at a finer scale are required to clarify the mechanisms by which glucosinolates affect herbivores; for example,

identifying whether the action of glucosinolates on herbivores is direct, or mediated by herbivore natural enemies. Therefore, I focused on an interaction between the specialist aphid, *Brevicoryne brassicae* and sinigrin glucosinolate, which was revealed to be important at both ecological scales by the population-wide survey. In addition, the effect of sinigrin on *B. brassicae* was consistent across all study years. Aphid colony dynamics were monitored on plants during the flowering period at three populations in Cornwall. This study revealed that colonies were significantly smaller on plants producing sinigrin compared to plants producing only butyl-derived glucosinolates. Experimental removal of all aphid natural enemies indicated that sinigrin (or a factor correlated with sinigrin production) had a direct effect on the growth rate of the colony. There was no evidence that a top-down effect of sinigrin, mediated by natural enemies, was important in the regulation of aphid colony size (Chapter 4).

In order to determine if this variation in aphid colony size between phenotypes led to a corresponding variation in plant fitness, I manipulated aphid colonies and their natural enemies on plants, and measured the effects on seed set (Chapter 5). If the fitness of different plant phenotypes varied in the presence or absence of aphids, this would indicate that genotype-by-environment interactions (Gillespie and Turelli 1989), mediated by herbivore presence or abundance, could be involved in maintaining the variation in heritable glucosinolate profile. The results from the study showed a negative effect of aphids on seed set, and a negative effect of sinigrin presence on seed set across all levels of aphid infestation. In other words, I found a significant environment effect (aphid presence) and a significant effect of plant genotype (the production of sinigrin, which corresponds to a functional allele at the *GSL-pro* locus (Magrath et al. 1994; Parkin et al. 1994; Mithen et al. 1995a), but there was no interaction between the two effects and consequently no evidence for genotype-by-

environment interactions. Nevertheless, the results demonstrate that the presence of aphids and plant chemical variation can have a significant impact on plant seed set.

To investigate whether the signal identified by the intensive study on sinigrin variation in the Cornish populations scaled up to a general impact of glucosinolate phenotype on seed set across all of the study populations I estimated the seed set for all marked wild cabbage plants that were flowering in 2008 (Chapter 6). Plant phenotype had a significant effect on seed set: a positive effect of each glucosinolate (sinigrin and progoitrin) individually, but a reduction in seed set in plants that produced the full range of aliphatic glucosinolates (Chapter 6).

Although there was no general trend in the effect of any herbivore species on seed set across populations, there was significant variation in the direction of the correlation between herbivore presence and glucosinolate profile within populations (Chapter 6). Only the effects of the most abundant herbivores (snails and *B. brassicae*) could be analysed within populations. The results showed that seed set was affected by the interaction between glucosinolate profiles and herbivore presence, however, the direction of the response was affected by the plant population. The fact that plant population mediates the effects of plant phenotype and herbivores on seed set indicates that environmental effects are highly important in interactions between plant fitness, plant defence chemistry and herbivores. This also highlights variation in plant seed set that cannot be explained by differential herbivory pressure. The population-wide seed set results compare favourably with the results obtained from the aphid colony manipulation presented in chapter 5. Both provide evidence for the influence of genotype (production of aliphatic glucosinolates) on seed set, and both indicate an importance of herbivores. However, the variation in seed set between the different glucosinolate phenotypes cannot be completely explained by herbivore presence and there is also an important influence of other environmental factors.

Estimating plant fitness is a complex issue and a reduction in seed production may have little effect on future plant abundance if plant populations are not seed limited, or if plants are long lived and can compensate for herbivore attack (reviewed in Maron and Crone 2006). Population models of perennial plants have shown that variation in survival is an important measure of plant fitness (Stokes et al. 2004). The survey in this thesis included only seed set in mature plants as a proxy for plant fitness and so if selection pressures have a greater effect on plant survival at an earlier stage (e.g. germination or seedlings) this would not have been detected by the survey methodology used in this thesis.

The surveys in this thesis were carried out utilizing naturally occurring variation in plant chemical profiles. The plants studied will show substantial differences in aspects of their phenotype other than aliphatic glucosinolate presence. Within the glucosinolate defence system there are many other elements which were not measured in this study and could also be influencing the responses of herbivores and affecting plant fitness. The effects of the glucosinolate defence system on herbivores may vary with the concentration of glucosinolates (Li et al. 2000; Kliebenstein et al. 2002), or the concentration or type of myrosinase and myrosinase associated proteins (Bones and Rossiter 1996; Stotz et al. 1999; Kliebenstein et al. 2002; Agrawal and Kurashige 2003). In addition, induction by herbivore species can have different effects on plant chemistry: the feeding mode of an insect can cause differential responses of the glucosinolate system (Kempema et al. 2007) and affect the nutritional quality of the whole plant (van Dam et al. 2004; Soler et al. 2005), leading to significant impacts in the interactions between plants and other herbivore species (Agrawal 2000). Although studies carried out in natural populations cannot control for all of these factors and can only present correlations between herbivores and plant chemistry, studies in natural populations are important in determining the influence of effects that have been defined

in controlled lab environment on plants in natural communities, and for describing interactions and responses that can be detected above the "noise" of highly variable natural environments that can then be used to direct more controlled common garden or reciprocal transplant experiments.

Ideal research programmes for investigating genotype-by-environment effects of plant chemistry and herbivores would involve common garden experiments using near isogenic plant lines that vary only in the alleles controlling sinigrin and prigoitrin biosynthesis. This would certainly be possible using *Arabidopsis thaliana* and could also be achieved in wild cabbage plants but would require several generations of backcrossing of rapid cycling wild cabbage plants.

WIDER IMPLICATIONS OF RESULTS AND FUTURE WORK

Contrary to previous studies in wild cabbage populations, the evidence presented here demonstrates that a number of herbivore species, several of which are economically important agricultural pests of *Brassica* crops, can respond to variation in plant chemistry, and this can lead to structuring of herbivore communities over two ecological scales.

Plants producing sinigrin appear to incur a cost in terms of seed set, relative to plants producing the full range of butyl-derived glucosinolates (Chapters 5 and 6). Only mature plants that were at the reproductive stage were investigated, so the advantages of producing sinigrin may be more evident at other stages in the plant life cycle. An interesting route for future work would be to investigate the proportion of each phenotype recruited into the population each year, and compare the frequencies of glucosinolate phenotypes in seedlings with that of mature plants. This may provide some indication of whether the early life stages are important in determining the

variation in plant phenotypes observed in the populations and may reveal a situation in which the production of sinigrin is selectively advantageous.

The cost of sinigrin production suggests that the maintenance of this glucosinolate is more likely to be driven by an herbivore species that is deterred by sinigrin. In chapters 4 and 5, aphids were tested as a candidate species that may be driving differential selection of glucosinolate phenotypes. The results showed there was evidence for components of top-down differential selection, however, a key piece of evidence is still lacking: that the relative fitness of the different phenotypes varies in the presence or absence of herbivore species. This was also true for the large scale survey of herbivore presence and the effect on seed set in plants across all 12 populations. This lack of signal was almost certainly due to the fact that two surveys per year was not sufficient to provide an estimate of herbivory pressure on plants, particularly in the case of some of the lepioptera species which can cause substantial damage to plant tissues but are present in relatively low abundance. More intensive surveys may help to reveal the effects of these less abundant herbivores on plants in natural populations. However, given the evidence in this thesis, it is not possible to rule out the role of genetic drift as a mechanism maintaining diversity.

Overall, there is evidence that glucosinolate phenotypes have an impact on plant seed production. Herbivores respond to the variation in secondary metabolites and the direction of these responses differs between species, such that a glucosinolate that deters one species may attract another. However, there was no clear signal that herbivore presence differentially affects the fitness of plant chemical phenotypes. There are four key points that suggests differential selection by herbivores merits further investigation as a mechanism maintaining secondary metabolite variation in natural populations. First, aliphatic glucosinolate variation structures the herbivore community in natural populations. Second, the effect of glucosinolates on herbivores varies spatially and

temporally. Third, the production of these glucosinolates has a strong genetic basis and previous studies revealed no evidence for genetic drift in the variation in glucosinolate profiles (Mithen et al. 1995b). Finally, better characterisation of the link between herbivore and plant fitness could reveal a consistent genotype-by-environment effect of herbivores and secondary chemistry on plant fitness.

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