

**ECOLOGICAL PATTERNS IN PLANT DEFENCE CHEMISTRY AND  
HERBIVORE RESPONSES IN NATURAL POPULATIONS OF *BRASSICA*  
*OLERACEA***

Submitted by:

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'...the plant-herbivore 'interface' may be the major zone of interaction responsible for generating terrestrial organic diversity.'

*Ehrlich & Raven (1964)*



*Brassica oleracea* flower by Emma Wood

## ABSTRACT

Relationships between two taxonomic kingdoms; plants and herbivorous insects, are hypothesized to be a major zone of interaction for generating current day biodiversity; and coevolutionary processes between these intricately linked organisms are hypothesized to maintain diversity in plant secondary chemistry. These metabolites play a key role in plant defence against herbivory and a high degree of intraspecific variation is observed at multiple ecological scales. However, the nature of selection maintaining variation in plant defence profiles is still a major question in evolutionary biology and ecology, and progress towards a deeper understanding is hampered by a lack of studies that take into account ecological context and the multivariate nature of plant defence phenotypes.

In this thesis, I employ sophisticated chemical analysis techniques to identify a suite of glucosinolate secondary chemicals, representing different biosynthetic pathways, in the wild cabbage, *Brassica oleracea*, in natural populations in the UK. I used model-based cluster analysis to explore patterns of association between individual glucosinolates, predicting that as simultaneous resource allocation to multiple defences is likely to be constrained; negative associations between defensive traits should be observed. However, results revealed positive associations between glucosinolates. Therefore co-expression of multiple defences may not be costly for this species.

Using this information in conjunction with herbivore surveys and experiments, I show that this mixture has the potential to shape patterns of herbivore abundance and host plant utilization: species-specific responses to variation in glucosinolate phenotypes are discovered at various ecological scales. Thus there is the potential for differential selection on plant chemotypes though species-specific attractions and aversions.

By conducting fine scale experiments with herbivore species, I also found that glucosinolate variation has an impact on the counter-adaptations that some brassica specialists have evolved: in order to optimally defend against their own natural enemies, *Brevicoryne brassicae* aphids sequestering glucosinolates from their host plants must do so selectively, and must choose plants whose chemical profile best matches this behaviour.

These findings show that glucosinolate profiles may be under natural selection by herbivores in wild populations, and that reciprocal evolution between these plants and their specialists may continue to promote diversity in secondary metabolites. Together these results highlight the complexity inherent in plant-insect interactions, the importance of field studies and generate a wealth of testable hypotheses for future work.

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## CHAPTER ONE

### PLANT SECONDARY METABOLITES: CHEMICAL WARFARE IN THE UNDERGROWTH

Intraspecific variation, the raw material required for evolutionary change (Stearns 1989), can have significant ecological consequences, particularly for species in close, reciprocal interactions (Bolnick et al. 2011). Intraspecific variation in plant traits for example, plays a major role in shaping the composition and diversity of the associated invertebrate community (Poelman et al. 2009), and interactions between plants and their herbivorous insects is hypothesized to be the major zone of interaction for generating current day terrestrial diversity (Ehrlich and Raven 1964). Identifying key plant traits mediating this relationship is therefore a major goal in evolutionary biology and ecology.

Plant secondary metabolites consist of some of the most highly variable traits seen in nature (Hartmann 1996): to date over 100,000 compounds have been identified and the estimated number is predicated to exceed 200,000 (Pichersky and Lewinsohn 2011). Secondary compounds were historically dismissed as simply the bi-products of primary metabolism. However, in 1959 Fraenkel recognised that secondary chemistry may have a primary, functional role in defence against natural enemies such as invertebrate and mammalian herbivores and pathogens (Rausher 2001). This provided an adaptive hypothesis, putting these compounds firmly on the agenda of evolutionary biologists and ecologists. Having provided an answer to the question “Why are there secondary metabolites in plants?” (Berenbaum and Zangerl 2008), the question of how and why such diversity should evolve and persist remained an evolutionary puzzle: given that herbivores and pathogens reduce the fitness of the plants on which they feed directly (Jarosz and Davelos 1995; Maron 1998;



Hawkes and Sullivan 2001; Strauss et al. 2002), and indirectly (Strauss et al. 1996; Mothershead and Marquis 2000); and if chemical defence deters or reduces enemy attack (Fraenkel 1959; Giamoustaris and Mithen 1995; Mauricio 1998; Dicke 2000) then plants should be under directional selection for increased defence. In the absence of costs, such directional selection should lead to the ‘best’ defence profile spreading to fixation in populations. However, extensive polymorphism in plant chemical phenotypes is observed at multiple ecological scales, within and among plant species (Berenbaum et al. 1991; Mithen et al. 1995b; Newton et al. 2009b; Rasmann and Agrawal 2011).

Five years later after Fraenkel’s seminal work, Erlich and Raven’s classic paper “ignited a scientific wildfire” (Janz 2011) when they proposed co-evolution between plants and their natural enemies as a mechanism for generating both extensive taxonomic diversity in secondary metabolites, and species radiations of phytophagous herbivores. They noted that taxonomic correspondence between groups of butterflies and the plants they feed on, was related to plant secondary chemistry, such that it often transcended taxonomic relatedness (Ehrlich and Raven 1964; Janz 2011). This correspondence fuelled their hypothesis of a coevolutionary arms race scenario, where increased plant chemical defence selects for enhanced insect ability to counter plant defences that, in a reciprocal manner, selects for the evolution of novel plant defences (Ehrlich and Raven 1964).

*The specialist/generalist paradigm as a mechanism for maintaining intraspecific variation in secondary metabolites.* At a micro-evolutionary level, this coevolutionary framework hypothesizes that the high, intraspecific variation in secondary metabolite concentration and composition observed in many plant species (for review, see Hartmann 1996) is maintained

through co-evolved chemical preferences and aversions of herbivorous species (Dethier 1954; Cornell and Hawkins 2003a). Specifically, it is predicted that generalist herbivores with relatively broad diets are effectively repelled by plant secondary compounds. Conversely specialist species, with narrower diet breadths and physiological and/or behavioural counter-adaptations to plant toxins, may be attracted to oviposit or feed by the presence of specific secondary compounds (van Meijden 1996). This scenario presents plants with a dilemma between defending against one type of attacker at the risk of attracting another. If the ratio of generalist to specialist herbivores within a plant population varies spatially and temporally, the resulting fluctuating selection pressures may maintain genetic variation in secondary profiles (Giamoustaris and Mithen 1995; Strauss et al. 2002; Lankau and Strauss 2007).

Whilst the importance of plant secondary metabolites to host plant selection by herbivores is widely accepted, there remains a lack of consensus regarding the patterns of selection exerted by insect herbivores on plant secondary profiles. For example as mentioned above specialists, often restricted to feeding on plants within the same family, are expected to exhibit positive responses to increasing chemical concentrations due to their numerous counter-adaptations (detoxification, excretion, etc. for review see Després et al. 2007). In contrast, generalist herbivores are expected to show negative responses to increasing concentrations of plant defence chemistry. However, it has been shown that generalist herbivores may also evolve adaptations to minimize plant defence activation or detoxify harmful toxins (Thies 1979; Whitman et al. 1992; Noret et al. 1999; Falk and Gershenson 2007). For example, a sulfatase enzyme for deactivating the “mustard-oil bomb” of cruciferous plants has been found in the snail *Cornu asperum* (Thies 1979) and the desert locust, *Schistocerca gregaria* (Falk and Gershenson 2007); an adaptation previously thought to be an evolutionary innovation restricted to the specialist moth *Plutella xylostella* (Ratzka et al. 2002).

In addition, specialists can also be negatively affected by particular plant metabolites. The small white butterfly *Pieris rapae*, a specialist on the glucosinolate-myrosinase system found in cruciferous plants, has evolved a nitrile-specifier gene which converts potentially toxic isothiocyanates into harmless nitriles (Wittstock et al. 2004; Winde and Wittstock 2011), and has been shown to be stimulated by increasing concentrations of specific compounds (Renwick et al. 1992; Huang and Renwick 1993; Renwick and Lopez 1999). However, the bi-products of some of these compounds have been shown to have detrimental effects on larval performance (Agrawal and Kurashige 2003; de Vos et al. 2008).

*Species-specific responses to plant chemistry could maintain variation in secondary profiles.*

Together, these results appear to challenge the specialist/generalist paradigm. However, consistency in the direction of responses to secondary profiles by herbivores, regardless of guild, could be sufficient to exert differential selection pressures on plant defence phenotypes. For example, natural populations of *Arabidopsis thaliana* exhibited polymorphism at loci controlling variation in secondary metabolite profiles, which also correlated with the distribution and long-term abundance of two specialist aphids across Europe (Züst et al. 2012). This pattern was consistent with the direction of differential selection exerted by these species in greenhouse experiments (Züst et al. 2012), even though they are both specialized on the chemical defences of their hosts. Therefore, there may be within-feeding guild variation in species responses to plant profiles, even between functionally similar herbivores. These may have the potential to select for specific chemical phenotypes, and thus drive evolutionary change in associated plant populations (Züst et al. 2012). Species-specific responses to secondary compounds have been reported for a number

of herbivores (Huang and Renwick 1993; Nielsen et al. 2001; Kempel et al. 2015), yet results are often highly variable depending on the nature of the study.

*The importance of ecological context.* Key problems have hampered the identification of selection pressures exerted by insect herbivores: first, plant-insect interactions are commonly studied in controlled conditions, far removed from the ecologically complex reality and second, herbivore responses to secondary metabolites are often examined in the context of single compounds. There are still relatively few field-based studies of secondary metabolite-mediated, plant-herbivore interactions (Louda and Rodman 1983; Moyes et al. 2000; Newton et al. 2009b; Newton et al. 2010; Züst et al. 2012; Goodey et al. 2015). Much of the data supporting differential responses of herbivores to secondary compounds comes from laboratory trials (Cornell and Hawkins 2003b). However, effects that are statistically significant in a controlled environment may not be ecologically significant (Gurevitch and Collins 1994), and there remains a relative paucity of evidence for the importance of secondary compounds in determining levels of herbivory on plants in natural populations.

In addition, the majority of studies typically explore plant-insect interactions in the context of secondary traits belonging to a single structural group or biochemical pathway. Whilst this is convenient, plants express secondary metabolites as complex mixtures, consisting of several structural types, which may vary in their biological action (Wink 2003). Even in plant species defended by one class of chemical, there are often many forms of these compounds (Berenbaum et al. 1991; Bones and Rossiter 1996; Bennett et al. 2013). Current methods in chemical analyses with highly sensitive detection capabilities now allow rapid, quantitative assessment of plant metabolite profiles (Mohn et al. 2007; Agerbirk and Olsen 2012),

yielding new potential for studying herbivore responses across a suite of compounds in wild populations.

A plant's defence phenotype is a combination of genetically determined, constitutive compounds, and inducible chemical traits that are heavily influenced by environmental stressors, such as herbivore damage (Baldwin 1999; for review, see Bennett et al. 2013).

Herbivores are therefore confronted with differences in relative concentrations of a suite of compounds from different structural groups, resulting in complex plant chemotypes determined by both genetic and environmental effects. Therefore, we need multivariate studies of secondary metabolite variation and herbivore responses in natural systems. My goal in this thesis is to address these issues using surveys of herbivore responses to complex secondary profiles in natural plant populations.

In order to do this, I exploit a plant species with a well-described defence system, the wild cabbage *Brassica oleracea*. Use of another brassica, the model plant *Arabidopsis thaliana*, has provided much information regarding the biosynthesis and genetic control of the taxonomically characteristic glucosinolate defence system of these plants (Kliebenstein et al. 2001c; Mewis et al. 2005; Rowe et al. 2008). However, *A. thaliana* is an annual which has the option of escaping herbivory through ecological disappearance (Chew and Courtney 1991). *B. oleracea* on the other hand, is a long-lived perennial with a potential life span of decades, providing discrete, reasonably predictable resources to which herbivores may become locally adapted (Traynier and Truscott 1991). These are not therefore, ecologically equivalent plant species, and the selection pressures driving diversity in their defence phenotypes may be very different.

*The glucosinolate-myrosinase system of the Brassicales.* The glucosinolate-myrosinase system of Brassicales (Matthäus and Luftmann 2000; Windsor et al. 2005) consists of structurally diverse compounds, classified according to their amino acid precursors and the types of modification to the R group (figure 1.1), into aliphatic, indolic or aromatic glucosinolates (Halkier and Gershenzon 2006. Glucosinolates are stored separately from hydrolysing enzymes known as myrosinases (Fahey et al. 2001; Halkier and Gershenzon 2006) which are brought into contact when tissue damage, such as that caused by herbivore feeding, breaks down compartmentalization (Halkier and Gershenzon 2006). Depending on the substrate structure, hydrolysis then converts the glucosinolate into various biologically active products including highly reactive isothiocyanates (Halkier and Gershenzon 2006; Winde and Wittstock 2011).

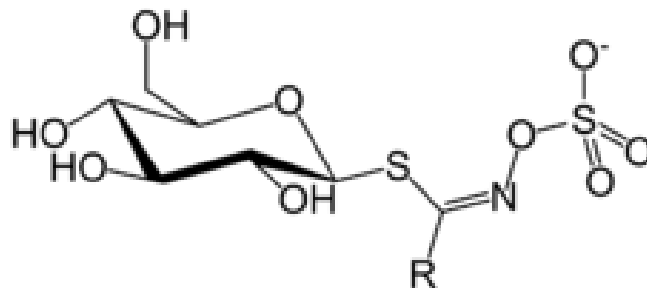


Figure 1.1. Basic glucosinolate structure showing variable R side chain group.

(adapted from Mahn and Reyes 2012)

*Glucosinolate-herbivore interactions.* The activation of glucosinolates upon plant tissue damage, and the toxic properties of their breakdown products, have long suggested that the major function of these compounds is to defend against natural enemies (Halkier and Gershenzon 2006). Numerous studies have demonstrated that glucosinolates exhibit outright toxicity to, or inhibit growth, deter feeding or oviposition, in a wide range of vertebrate and invertebrate plant enemies (reviewed in Giamoustaris and Mithen 1995; Halkier and Gershenzon 2006; Textor and Gershenzon 2009). Conversely, as with other secondary metabolites, the same compounds may serve as host location and acceptance cues for many specialized herbivores (reviewed in, Textor and Gershenzon 2009; Müller et al. 2010).

However, a recent study investigating patterns of host utilization across wild populations of *Brassica oleracea* in response to qualitative variation in sinigrin and progoitrin, found a positive association between snails, generalist herbivores, and sinigrin (Newton et al. 2010): this result hardly fits the prediction that generalists should avoid defence metabolites. The same authors also reported a positive relationship between sinigrin and the presence of the brassica specialist butterfly *Pieris brassicae* (Newton et al. 2009b), and therefore concluded that herbivores showing similar responses to glucosinolates could not be grouped into generalists and specialists. Furthermore, a survey of the literature describing herbivore responses to individual glucosinolates revealed substantial variation in the direction of response, with positive, negative and equivocal responses often found for the same species (table 1.1). These inconsistencies in reported herbivore responses to glucosinolates may be due to the tendency to consider plant-herbivore interactions with single compounds.

Table 1.1 Studies showing the effect of different glucosinolates on the behaviour and performance of a subset of specialist insect herbivores and one generalist. Studies were included if they investigated herbivore responses to individual, rather than total, glucosinolates

<b>Insect</b>	<b>Glucosinolate compound<sup>a</sup></b>	<b>Type of study<sup>b</sup></b>	<b>Behaviour measured</b>	<b>Effect<sup>c</sup></b>	<b>Plant</b>	<b>Ref</b>
<i>Brevicoryne brassicae</i>	Gluconapoleiferin <sup>a</sup>	G	Intrinsic rate of increase	+	<i>Brassica spp. and cultivars</i>	1
	Progoitrin <sup>a</sup>	F, G	Abundance, intrinsic rate of increase	+, -, 0	<i>Brassica oleracea, Brassica spp. and cultivars</i>	2, 3, 4, 1
	Gluconapin <sup>a</sup>	F	Abundance	+, 0	<i>B.oleracea</i>	4, 2
	Sinigrin <sup>a</sup>	F, L, C.G	Intrinsic rate of increase, feeding intensity, abundance, pupal mass	+, -, 0	<i>Brassica spp. and cultivars, Brassica nigra, B.oleracea</i>	1, 5, 2, 6, 7, 8, 9, 3, 4
	Glucobrassicin <sup>a</sup>	G	Intrinsic rate of increase	+	<i>Brassica spp. and cultivars</i>	1
	Glucobrassicin <sup>i</sup>	F	Abundance	-, 0	<i>B.oleracea</i>	4, 2, 10
<i>Pieris rapae</i>	Progoitrin <sup>a</sup>	G, F	Development time, pupal mass, abundance	+, 0	<i>B.oleracea, Brassica cultivars</i>	2, 11, 12, 9
	Gluconapin <sup>a</sup>	G, F	Development time, pupal mass, abundance	0	<i>B.oleracea, Brassica cultivars</i>	2, 11, 12
	Sinigrin <sup>a</sup>	L,G, F	Oviposition, feeding, development time, pupal mass, abundance	+, 0	Leaf wash, <i>B. oleracea cultivars</i>	13, 14, 15, 2, 11, 12, 9
	Glucocheirolin <sup>a</sup>	L	Oviposition	+	Leaf wash	16
	Glucobrassicin <sup>i</sup>	L, G	Oviposition, sensilli development time, pupal mass, abundance	+, 0	Leaf wash, <i>B. oleracea cultivars, B.oleracea,</i>	13, 14, 16, 2, 11, 12
	Hydroxyglucobrassicin <sup>i</sup>	G	Development time, pupal mass	0	<i>B.oleracea, Brassica cultivars</i>	11, 12
<i>Pieris brassicae</i>	Progoitrin <sup>a</sup>	F	Abundance	0	<i>B. oleracea</i>	3, 9



	Gluconapin <sup>a</sup>	F	Abundance	<b>0</b>	<i>B. oleracea</i>	2
	Sinigrin <sup>a</sup>	G, F	Abundance, growth rate	+, -, <b>0</b>	<i>Brassica nigra</i> , <i>B. oleracea</i>	2, 16, 9
	Glucobrassicin <sup>i</sup>	L, F	Oviposition	+, <b>0</b>	Leaf washes, <i>B. oleracea</i>	18, 2
	Hydroxyglucobrassicin <sup>i</sup>	G	Growth rate	+	<i>B. nigra</i>	17
Snails	Sinigrin <sup>a</sup>	F, C.G	Abundance	+, -, <b>0</b>	<i>B. oleracea</i> , <i>B. nigra</i>	6, 9, 3
<i>Myzus persicae</i>	Gluconapin <sup>a</sup>	G	Intrinsic rate of increase	-	20 Brassica accessions	1
	Glucobrassicin <sup>i</sup>	G	Intrinsic rate of increase	+	20 Brassica accessions	1
	Hydroxyglucobrassicin	G	Fecundity	-	<i>Arabidopsis thaliana</i> and purified extracts	19

<sup>a</sup> abbreviations used: <sup>a</sup>: Aliphatic compounds; <sup>i</sup>: Indole compounds

<sup>b</sup> C.G: Common garden; G: Greenhouse; F: Field study; L: Laboratory

<sup>c</sup> +: positive correlation; -: negative correlation; **0**: no correlation

References: <sup>1</sup>Cole 1997; <sup>2</sup>Moyes et al. 2000; <sup>3</sup>Newton et al. 2010; <sup>4</sup>Goodey et al. 2015; <sup>5</sup>Gabrys and Tjallingii 2002; <sup>6</sup>Lankau 2007; <sup>7</sup>Lankau and Strauss 2007; <sup>8</sup>Pratt et al. 2008; <sup>9</sup>Newton et al. 2009; <sup>10</sup>Mewis et al. 2006; <sup>11</sup>Gols et al. 2008; <sup>12</sup>Poelman et al. 2009; <sup>13</sup>Renwick et al. 1992; <sup>14</sup>Huang and Renwick 1993; <sup>15</sup>Renwick and Lopez 1999; <sup>16</sup>Stadler et al. 1995; <sup>17</sup>Smallegange et al. 2007; <sup>18</sup>van Loon et al. 1992 <sup>19</sup>Kim and Jander 2007

1 *Natural variation in plant glucosinolate profiles.* The glucosinolate phenotype of a plant is  
2 determined by both genetic and environmental effects: aliphatic glucosinolate type and concentration  
3 are under strong genetic control and are highly heritable (Kliebenstein et al. 2001b), resulting in  
4 variable, yet relatively predictable phenotypes between and among populations (Mithen et al.  
5 1995b). However, aromatic and indole glucosinolates are highly plastic and may be rapidly induced  
6 in response to abiotic and biotic stressors in a plant's local environment (Agrawal et al. 2002;  
7 Agerbirk et al. 2009).

8

9 The substantial variation in aliphatic glucosinolate type and quantity documented for *B. oleracea*,  
10 both within and among populations (Mithen et al. 1995b; Moyes et al. 2000; Bidart-Bouzat and  
11 Kliebenstein 2008; Newton et al. 2009b), makes this species an ideal study system in which to  
12 investigate secondary metabolite-mediated interactions with herbivores. In the United Kingdom, wild  
13 populations of *B. oleracea* occur on the coasts of Kent, Dorset, Devon, Cornwall, Glamorgan and  
14 Gwynedd (Snogurup et al. 1990). Evidence suggests that glucosinolate polymorphism in *B. oleracea*  
15 phenotypes is unlikely to be maintained by stochastic effects: differentiation between natural plant  
16 populations, in terms of allele frequencies at loci underlying defence-related traits, are greater than  
17 those predicted by genetic drift or founder effects (Zanetto and Kremer 1995; Mithen et al. 1995b;  
18 Kirk et al. 2010). Furthermore, geographic analyses of genetic variation in several related plant  
19 species have revealed clear genetic signals of local adaptation in defensive traits (Linhart and Grant  
20 1996), results which are further supported by reciprocal transplant experiments, in which defence  
21 phenotypes of home plants generally outperform those transplanted from other populations (Leimu  
22 and Fischer 2008; Fournier-Level et al. 2011). Finally, whilst the abiotic environment is clearly an  
23 important influence (Simon et al. 2011; Martínez-Ballesta et al. 2013), the large phenotypic effects

24 of loci underlying secondary traits are more indicative of biotic selection in plants (Louthan and Kay  
25 2011).

26 *Scope of thesis.* The remaining six chapters of this thesis aim to address some of the outstanding  
27 problems discussed in earlier sections here, in the hope of increasing our understanding of the nature  
28 of plant-insect interactions mediated by plant defence chemistry in natural populations.

29

30 **Chapter two** describes the process of developing a rapid and repeatable glucosinolate extraction  
31 methodology, which permitted identification and quantification of glucosinolates for a large number  
32 of plants. For subsequent chapters, we continue to quantify as many glucosinolates as possible.  
33 However, particular compounds, such as neoglucobrassicin, are often present at extremely low  
34 concentrations and were undetectable for plants in some populations. **Chapter three** uses this  
35 method to identify and measure the full diversity of aliphatic and indole glucosinolate chemistry in  
36 plants from spatially distinct wild populations, representing five regions in the South West of  
37 England. Using model-based clustering techniques, we look for evidence of trade-offs among  
38 different defence types, which is widely assumed to be a key mechanism maintaining diversity in  
39 plant secondary profiles.

40

41 With our increased knowledge of the wild glucosinolate phenotype, we begin to explore links  
42 between variation in glucosinolate profiles and the abundances of generalist and specialist herbivores  
43 in **chapter four**. We find that responses to glucosinolate phenotypes vary with herbivore species,  
44 regardless of feeding category. Therefore, we go on to explore pairwise interactions between  
45 glucosinolates and specific, specialist herbivores, using theoretical optimality frameworks.

46 For example, **chapter five** investigates how intraspecific variation in glucosinolate profiles affects  
47 what is predicted to be a tight link between female oviposition preference and larval performance, in  
48 the large white butterfly, *Pieris brassicae*. **Chapter six**, published in *Journal of Chemical Ecology* in  
49 2015, highlights how a specialist aphid sequesters key glucosinolates from its host optimally, and  
50 does not simply passively reflect host plant chemistry. **Chapter seven** explores this fascinating result  
51 further using a theoretical, optimal defence framework, showing that higher concentrations of host  
52 glucosinolates were observed for the aphid morph of highest reproductive value.

53 Finally, a general discussion is provided in **chapter eight**, where we also propose avenues of future  
54 research using this promising system.

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## CHAPTER TWO

67

68 GEOGRAPHIC AND BIOCHEMICAL PATTERNS OF VARIATION IN PLANT DEFENSE

69 PROFILES IN WILD POPULATIONS OF *BRASSICA OLERACEA*

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## ABSTRACT

74 Models of the evolution and maintenance of plant secondary metabolites assume that investment in  
75 defence incurs costs, diverting limited resources away from key fitness processes of growth and  
76 reproduction. Therefore according to classic theories of plant defence, trade-offs are predicted  
77 between different defence types. An increasing number of studies refute this, reporting no  
78 association between chemical defence traits, but the majority of studies have investigated patterns of  
79 variation in secondary metabolite profiles under controlled conditions. If trade-offs are key  
80 mechanisms for maintaining secondary metabolite diversity, then it is important to establish their  
81 occurrence in natural populations. Here we survey the multivariate glucosinolate defence phenotypes  
82 of wild cabbage, *Brassica oleracea*, representing natural populations from five counties in the UK.  
83 We compare patterns of association among the defence traits of wild plants to those of plants grown  
84 in a common garden to remove the effects of environmental covariates. Instead of trade-offs among  
85 glucosinolates, we found positive associations within and among glucosinolate structural groups,  
86 suggesting that the production of multiple defences is not constrained in this plant species. Patterns  
87 of association were consistent for both wild and common garden plants, therefore associations are  
88 not due to clinal changes in resource availability or allocation. However, correlations for common  
89 garden plants were weaker, suggesting that environmental covariates, for example variation in soil  
90 nutrients, play at least some role in generating positive associations. These results add to a growing  
91 body of evidence reporting a lack of trade-offs between plant defences, suggesting other mechanisms  
92 may be maintaining variation in secondary metabolite profiles.

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94

## INTRODUCTION

95 A central goal in the study of the evolution and ecology of plant-insect interactions is to explain and  
96 predict patterns of genetic, phenotypic and geographic variation in plant defence traits (Stamp 2003;  
97 Poelman et al. 2008b). Plants produce a diverse array of chemical defences, known as secondary  
98 metabolites, which confer various types of resistance to natural enemies, and mediate complex  
99 interactions with the associated invertebrate community (Price et al. 1980; Díaz and Cabido 2001;  
100 Poelman et al. 2009). Substantial genetic variance has been documented for wild plants at a range of  
101 ecological scales (Adler et al. 1995; Hemming and Lindroth 1995; Mithen et al. 1995b; Kliebenstein  
102 et al. 2001b). However, the majority of studies typically measure variation of traits belonging to one  
103 structural group or biochemical pathway.

104

105 In reality, plants express secondary metabolites as complex mixtures, consisting of several structural  
106 types, which may vary in their biological action (Wink 2003). Even in plant species defended by one  
107 class of chemical, there are often many forms of those compounds (Berenbaum et al. 1991; Bones  
108 and Rossiter 1996; Bennett et al. 2013). Furthermore, a plant's defence phenotype is a combination  
109 of genetically determined, constitutive compounds and inducible chemical traits that are heavily  
110 influenced by environmental stress, such as herbivore damage (Baldwin 1999; for review, see  
111 Bennett et al. 2013). Thus when attempting to characterise plant defence profiles, it is more relevant  
112 to consider suites of potentially interacting compounds (Agrawal and Fishbein 2006) that are the  
113 product of genetic background and environmental variation.

114

115 Describing the multivariate defence phenotype, and uncovering patterns of association between  
116 individual traits, is of fundamental importance for understanding the selective forces generating  
117 chemical diversity (Agrawal and Fishbein 2006). Conventional theories for explaining the  
118 considerable variation in composition and concentration of secondary profiles, and the lack of fixed  
119 ‘optimal’ defence genotypes, are underpinned by the assumption that defences are costly to produce  
120 (Coley et al. 1985; Bazzaz et al. 1987a; Strauss et al. 2002). Plants have limited resources to support  
121 the energetically demanding processes of growth, reproduction and defence (Herms and Mattson).  
122 Furthermore, primary and secondary metabolic pathways share common amino acid precursors  
123 (Halkier and Gershenzon 2006), thus the synthesis of secondary products may compete with a  
124 primary processes requiring the same substrate (Jones and Hartley 1999). Such energetic limitations  
125 result in trade-offs between somatic and defence related traits (Herms and Mattson 1992).

126

127 Trade-offs and constraints are inherent in the most prominent theories of plant defence, including  
128 optimal defence theory (McKey 1974; Rhoades 1979), apparency theory (Feeny 1976), the growth-  
129 differentiation balance hypothesis (Herms and Mattson 1992) and the resource availability  
130 hypothesis (Coley et al. 1985). In all of these theoretical frameworks, trade-offs are predicted  
131 between defence chemicals of different structural types, and between constitutive and inducible  
132 defences (Herms and Mattson 1992), many of which share common precursors and exact substantial  
133 resource allocations costs (Bekaert et al. 2012). Thus simultaneous allocation to multiple defences is  
134 predicted to be constrained, and should result in negative associations between different defensive  
135 traits (Coley et al. 1985).

136

137 However, supporting evidence, expressed through negative correlations between defence traits (Roff  
138 and Fairbairn 2007), is very mixed and several recent reviews and meta-analyses have found little  
139 support for such costs (Bergelson and Purrington 1996; Koricheva et al. 2004; Leimu and Koricheva



140 2006). Indeed, many studies have reported significant positive correlations among defence traits  
141 (Hougen-eitzman and Rausher 1994; Mitchell-Olds et al. 1995; Agrawal et al. 2007).

142 There are several explanations for why traits expected to be negatively associated might exhibit  
143 positive relationships. First, whether positive or negative associations between traits are observed in  
144 wild plant populations may depend on environmental variation (Sgrò and Hoffmann 2004). There is  
145 growing evidence that changes in conditions can influence interactions among life history traits, as  
146 well as genetic variance in the traits themselves (for review, see Sgrò and Hoffmann 2004). For  
147 example, correlations between traits may be positive in environments where resources are abundant  
148 (Sgrò and Hoffmann 2004), or may simply reflect an underlying cline in resource availability.  
149 Second, individuals may differ not only in allocation of resources but also in acquisition. If  
150 acquisition is more variable than allocation, positive correlations will arise as some individuals  
151 within a population command more resources than others (Reznick et al. 2000), and thus produce  
152 more defences by simply being ‘better’ plants. Furthermore, allocation to defence may also incur  
153 indirect ‘ecological costs,’ such as reduced competitive ability or tolerance to herbivory, that are only  
154 evident when plants are growing in an ecologically relevant context (e.g., van Dam and Baldwin  
155 1998; Cipollini 2002).

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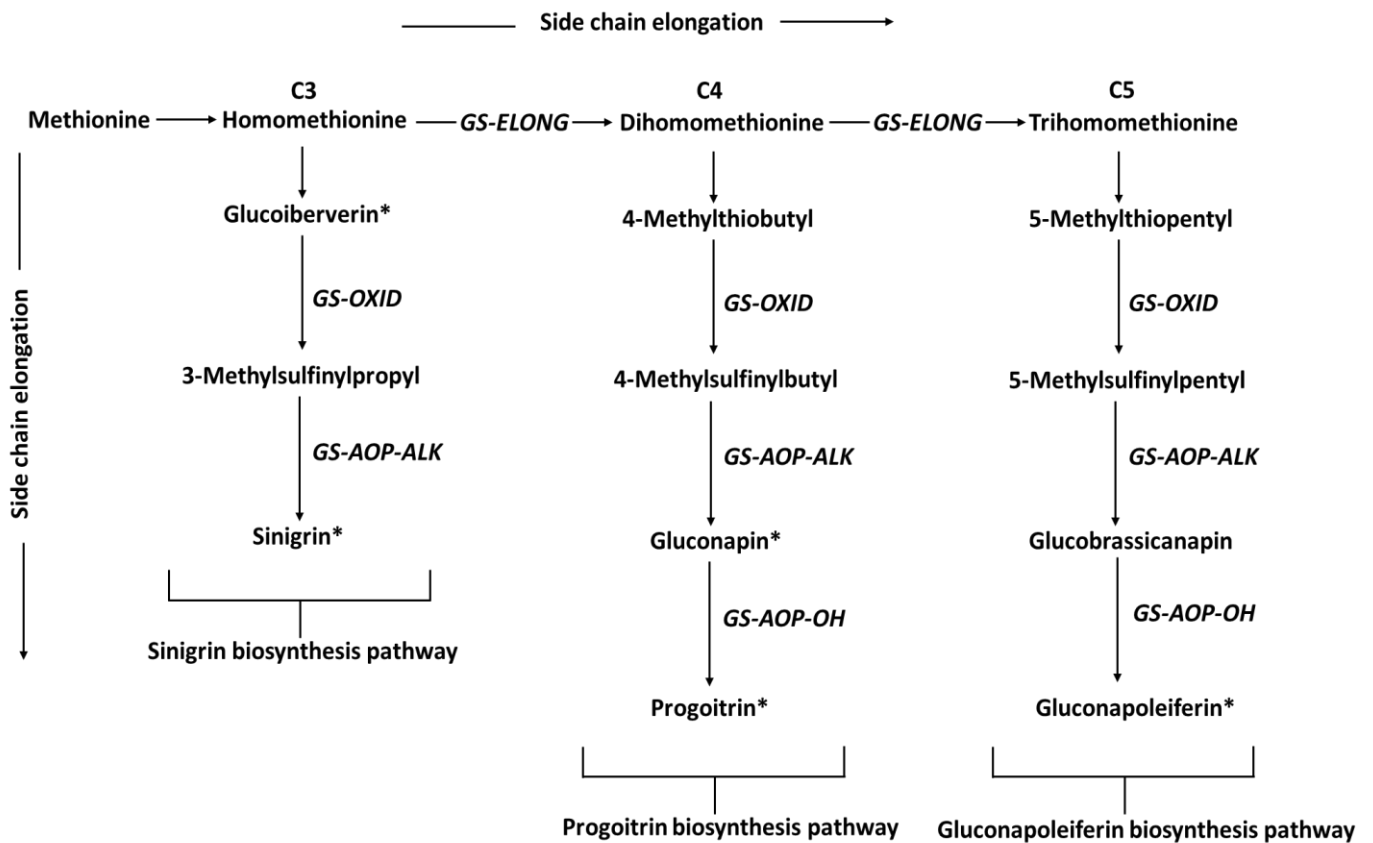
157 If trade-offs are key mechanisms for maintaining secondary metabolite diversity, then it is important  
158 to establish their occurrence in natural populations. In this study, we aim to identify secondary  
159 metabolite phenotypic diversity across multiple natural plant populations, specifically investigating  
160 patterns of association between constitutive and inducible defences, and between defence chemicals  
161 of different structural types. We further aim to verify results observed in wild plants by comparing  
162 trends to plants reared in a common garden environment, in order to remove any underlying clines in  
163 resource availability.

164 To address this, we exploit a plant species with a well-described defence system, the wild cabbage  
165 *Brassica oleracea*. Like most species in the Brassicales, *B. oleracea* contains sulphur and nitrogen  
166 containing secondary metabolites known as glucosinolates (Matthäus and Luftmann 2000; Windsor  
167 et al. 2005). When insect herbivores feed on the plant, tissue damage brings intact glucosinolate  
168 compounds in contact with an activated enzyme, myrosinase, which catalyses the production of  
169 highly toxic hydrolysis breakdown products (Bones and Rossiter 1996; Halkier and Gershenzon  
170 2006). The biological role of glucosinolates depends on chemical concentration, structure and group  
171 (Giamoustaris and Mithen 1995). For example, although glucosinolates share a common glycone  
172 moiety, compounds vary in the structure of an aglycone side chain which is derived from a variety of  
173 different amino acids (Bones and Rossiter 1996; Mithen 2001). This determines whether a compound  
174 belongs to a constitutively expressed group, known as aliphatic chemicals (figure 2.1), or to a group  
175 of inducible compounds known as indole glucosinolates (figure 2.2) (Mithen 2001). Glucosinolate  
176 profiles of wild populations of *B. oleracea* have been shown to be highly variable (Mithen et al  
177 1995), and this variation appears to be mainly driven by the presence or absence of two aliphatic  
178 compounds, sinigrin and progoitrin (Newton et al. 2009b). No studies to date have investigated  
179 patterns of covariance between glucosinolate types in *B. oleracea*.

180

181 Here we survey *B. oleracea* plants from spatially distinct wild populations representing five regions  
182 in the South West of England. We identify and measure the full diversity of aliphatic and indole  
183 glucosinolate chemistry and investigate patterns of covariance between them. Based on previous  
184 findings, we expect significant geographic diversity in glucosinolate profiles, driven by variation in  
185 the expression of sinigrin and progoitrin. We hypothesize that, according to classic plant defence  
186 theory, there will be negative phenotypic correlations between constitutive and inducible  
187 glucosinolates, and between different glucosinolate structures which share a common precursor.

188 However, the concentration and pattern of expression of glucosinolates are strongly affected not only  
189 by genotype, but also by the environment (Valente Pereira et al. 2002). Glucosinolates represent as  
190 much as 30% of the total sulphur content of plants, thus glucosinolate accumulation will be linked to  
191 the sulphur content of the entire plant (Falk et al. 2007). For example, variation in available sulphur  
192 and has been shown to influence not just the total glucosinolate concentration a plant produces, but  
193 also has differential effects on constitutive versus indole compounds (Rosen et al. 2005; Falk et al.  
194 2007). Thus, in order to remove the effects of environmental covariates, we compare results with  
195 those of plants from the same wild populations, reared in a common garden environment from 2007.  
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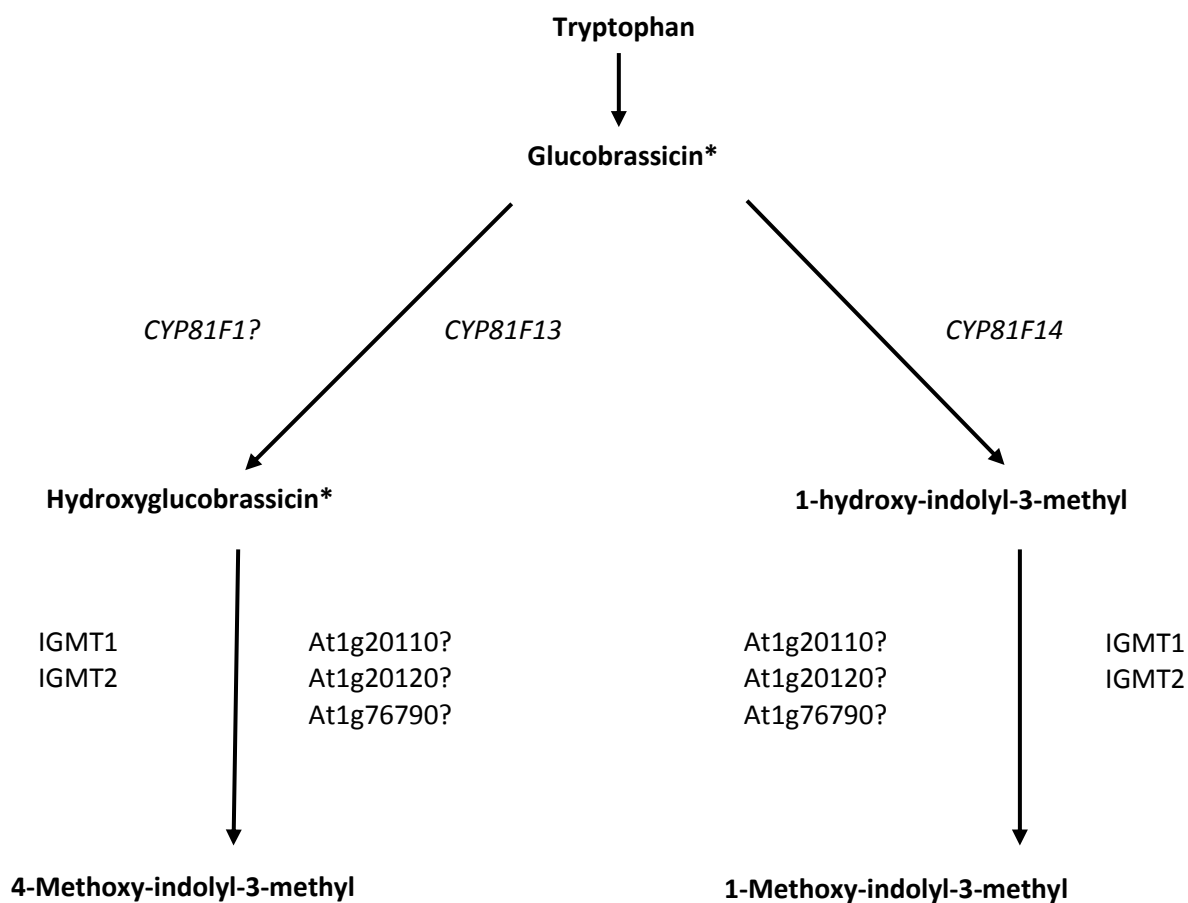
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Figure 2.1. Biochemical pathways, genetic loci and alleles that yield structural diversity of aliphatic glucosinolates in *Brassica oleracea* (adapted from Mithen 2001; Kliebenstein et al. 2001b; Halkier and Gershenzon 2006; Züst et al. 2012). Common names in bold; chemical names in normal font or bracketed under common names; loci and alleles in italics. A methionine backbone yields an elongated carbon chain (transitions from C3 to C4 to C5 chain length mediated by alleles at locus *GS-ELONG*, Methylthioalkylmalate synthases (Kliebenstein et al. 2005) with side group modifications yielded by alleles at loci *GS-OXID* and *GS-AOP* (glucosinolate (S) oxygenase (Kliebenstein et al. 2001c) and 2-oxo acid-dependent dioxygenases (Halkier and Gershenzon 2006) respectively. In each pathway, allele *GS-AOP-ALK* yields the non-hydroxylated end product. Further modification of the C4 and C5 side chains is yielded by *GS-AOP-OH* to produce progoitrin and gluconapoleiferin. Asterisked glucosinolates are those assayed in this study.

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Figure 2.2. Biochemical pathways, genetic loci and alleles that yield structural diversity of indole glucosinolates (from Pfalz et al. 2011). Enzyme functions are inferred from a combination of *Arabidopsis thaliana* T-DNA insertions in *Nicotiana benthamiana* and biochemical assays, and are shown above arrows. Further gene products that *may* catalyse the same reaction are shown below arrows. *CYP81Fs* carry out hydroxylation reaction of the glucosinolate ring, converting glucobrassicin either to hydroxyglucobrassicin or to 1-Methoxy-indolyl-3-methyl (Pfalz et al. 2011). Indole glucosinolate methyltransferase's (IGMT1 and IGMT2) convert these hydroxyl intermediates further to 1-Methoxy-indoyl-3-methyl or 4-Methoxy-indoyl-3-methyl respectively (Pfalz et al. 2011).

Asterisked glucosinolates are those assayed in this study.

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## MATERIALS AND METHODS

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*Study system.* Structural variation of aliphatic compounds is under heritable, genetic control by four major loci (figure 2.1) (Mithen et al. 1995a; Giamoustaris and Mithen 1996), which function epistatically, creating a modular system which enables the plant to generate different structural profiles (Kliebenstein 2009). Indole glucosinolate type and concentration is under weaker genetic control (figure 2.2) (Kliebenstein et al. 2001a; Pfalz et al. 2011), and variation appears to be predominantly driven by environmental effects (Agerbirk et al. 2009).

*Wild Plant Surveys.* Plants were randomly sampled from ten, spatially distinct populations in five counties in the UK: Kent, Glamorgan, Dorset, Devon and Cornwall. 50 plants per population were sampled and from each plant, five intact leaves were excised; representing a range of leaf sizes and ages, and immediately flash frozen in liquid nitrogen. Samples were collected during May-June 2013, with the exception of plants from Dorset populations which were collected during June 2014.

*Common Garden.* In 2009, seeds were collected from randomly selected wild plants; 50 plants per spatially distinct population were sampled from Cornwall, Dorset and Devon. Seeds were germinated and seedlings reared on benches in a greenhouse under ambient light supplemented with fluorescent lighting and watered daily. Pots were placed randomly on benches and randomly moved to different locations on the benches to minimize micro environmental effects. After three months plants were randomly transplanted into a south facing field, cleared of all other vegetation. The plot was weeded of other plants recruited into the plot at least three times a year. Leaf samples for chemical analyses were collected in May-June 2013, using the same protocol as for wild plant surveys.

231 *Extraction and Analysis of Intact Glucosinolates.* Plant material was lyophilized to dryness, and  
232 ground to a powder using tungsten beads with a diameter of 2 mm and a vibrating-ball micro mill  
233 (based on the Retsch MM300 ball mill). Tissue disruption was carried out for 4 min at a vibration  
234 frequency of 25 s<sup>-1</sup>. A 10mg aliquot of plant leaf tissue was transferred into 1.5 ml microcentrifuge  
235 tubes. Thermal degradation of glucosinolates has been observed at temperatures over 50°C (Mohn et  
236 al. 2007), therefore extraction took place in cold, 80% methanol (Goodey et al. 2015): 400 µl of  
237 extraction medium (80% methanol containing internal standards umbelliferone, 7.2 µg/ml, and  
238 linamarin 1.25 µg/ml) was added to plant samples. Lyophilized tissue samples were incubated on ice  
239 for 30 minutes with vortex mixing every ten minutes, followed by 15 minutes of sonication. After  
240 centrifugation(10 min at 16,100 x g, 4 °C), the supernatant was decanted and filtered through a 0.45  
241 µm (PVDF) syringe filter (Chromacol, Welwyn Garden City, UK).

242

243 To generate a list of glucosinolates compounds present in the *B. oleracea* populations (Table 2.1),  
244 metabolite profiling was performed using a QToF 6520 mass spectrometer (Agilent Technologies,  
245 Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. Glucosinolates fragment  
246 to produce sulfate product ions that can be used as identifiers for glucosinolates (Barbieri et al. 2008;  
247 Fabre et al. 2007; Mellon et al. 2002;Rochfort et al. 2008; Tian et al. 2005). Sample extract (5 µl)  
248 was loaded onto a Zorbax StableBond C18 1.8 µm, 2.1 x 100 mm reverse phase analytical column  
249 (Agilent Technologies, Palo Alto, USA). Mobile phase A comprised 5% acetonitrile with 0.1%  
250 formic acid in water, and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The  
251 following gradient was used: 0 min – 0% B; 3 min – 0% B; 7 min – 20% B; 20 min – 100% B;  
252 30 min – 100% B; 31 min – 0% B. The flow rate was 0.25 ml min<sup>-1</sup> and the column temperature was  
253 held at 35 °C for the duration. The source conditions for electrospray ionization were as follows: gas  
254 temperature was 325 °C with a drying gas flow rate of 9 liter min<sup>-1</sup> and a nebuliser pressure of 35  
255 psig. The capillary voltage was 3.5 kV. The fragmentor voltage was 115 V and skimmer 70 V.

256 Scanning was performed using the auto MS/MS function. Survey scan rate was 4 scans s<sup>-1</sup> and  
257 MS/MS scan rate was at 3 scans sec<sup>-1</sup> with a sloped collision energy of 3.5 V/100 Da with an offset  
258 of 5 V.

259

260 Quantitative glucosinolate analysis was performed using multiple reaction monitoring (MRM) on an  
261 Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto,  
262 USA). The HPLC system was the same as that used for QToF analyses. Sample extracts (15 µl) were  
263 loaded onto a Zorbax Eclipse Plus C18 3.5 µm, 2.1 x 150 mm reverse phase analytical column  
264 (Agilent Technologies, Palo Alto, USA) with fragmentor voltage and collision energies optimized  
265 for each compound (Table 2.1). The following gradient was used: 0 min – 0% B; 1 min – 0% B;  
266 5 min – 20% B; 20 min – 100% B; 25 min – 100% B; 27 min – 0% B. QQQ source conditions were  
267 as follows: gas temperature 350°C, drying gas flow rate 9 liter min<sup>-1</sup>, nebuliser pressure 35 psig,  
268 capillary voltage ±4 kV.

269

270 Glucosinolates were identified by accurate mass and the presence of characteristic product ions  
271 (Table 2.1) (Barbieri et al. 2008; Fabre et al. 2007; Mellon et al. 2002; Velasco et al. 2008). The  
272 presence of sinigrin and progoitrin were further confirmed by comparing retention times and ion  
273 fragmentation patterns with pure standards (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany  
274 and Merck KGaA, Darmstadt, Germany). In the absence of standards for all the glucosinolates,  
275 concentrations are expressed as peak areas normalized by peak area of the internal standard,  
276 linamarin.

277

278



279 *Statistical Analyses.* Statistical analyses were performed in R 3.0.2 (R Core Team 2013). All  
280 analyses were performed using log-transformed glucosinolate concentrations: this transformation  
281 normalized the residuals and satisfied the homogeneity of variance assumption for standard  
282 parametric statistical tests. Glucosinolate concentrations were scaled to have zero mean and unit  
283 standard deviation.

284

285 *Model-based clustering of wild and common garden glucosinolate profiles.* Model-based clustering  
286 is a powerful tool for studying the variation of multiple traits simultaneously and identifying  
287 homogenous groups. In this approach, the assumption is made that data are distributed according to  
288 a mixture of Gaussian probability distributions, with different distributions representing different  
289 clusters. Parameters defining a cluster consist of a mean vector that determines the centre of a  
290 cluster, and a covariance matrix that determines the cluster's geometry. Covariance matrices are  
291 parameterized in terms of eigenvalue decomposition, thus characteristics of an individual cluster can  
292 be allowed to vary in shape, volume or orientation, or can be constrained to be the same for all  
293 clusters.

294

295 Each combination of differently parameterized covariance matrices and different cluster number,  
296 corresponds to a separate, Bayesian probability distribution (Fraley and Raftery 2002). Therefore  
297 choosing the best clustering algorithm and correct number of clusters is reduced to a model selection  
298 problem (Bouveyron and Brunet-Saumard 2014). Model selection is determined by examination of  
299 associated BIC scores with a large BIC indicating strong evidence for the corresponding covariance  
300 matrix parameter and cluster number (Fraley et al. 2012). Typically, BIC scores with a difference  
301 greater than ten offer good support for selecting one model over another (Fraley and Raftery 2002).

302 We performed model-based cluster analysis on wild and common garden data sets separately,  
303 implemented in the R package mclust (Fraley et al. 2012).

304

305 *Gaussian dimension reduction of wild and common garden glucosinolate clusters.* In order to  
306 identify glucosinolate features which may be of particular importance, we investigated the subspace  
307 that captured the clustering information contained within the data. We used the mclust function  
308 mclustDR (Fraley et al. 2012), which performs dimension reduction by reducing clusters through a  
309 linear set of combinations, or directions, of the original glucosinolate variables. As above, this  
310 dimension reduction was performed separately for wild and common garden data sets.

311

312 *Geographical patterns of wild plant glucosinolate clusters.* The effect of geography on the frequency  
313 of plants belonging to each glucosinolate cluster was tested using contingency table analysis.

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## RESULTS

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330 First we present the results of model-based clustering of glucosinolate from wild plants which reveal  
331 patterns of positive association between compounds within and between glucosinolate pathways. We  
332 then compare these patterns with results from the analysis of common garden plants, which exhibit  
333 similar trends. We follow this with dimension reduction, and show that the aliphatic compounds  
334 sinigrin, progoitrin and gluconapin are compounds whose variation is of particular significance to  
335 glucosinolate clustering. Finally, analysis of the frequency of plants belonging to each glucosinolate  
336 cluster shows that geography does not fully explain phenotypic variation.

337

338 *Glucosinolates*. We quantified nine glucosinolates belonging to aliphatic and indole glucosinolate  
339 groups (table 2.1). The biosynthesis pathway of glucocheirolin and glucocapparin is currently  
340 unknown for *B. oleracea*.

341

342

343 Table 2.1. Transitions and data acquisition parameters used in HPLC-MSMS for quantification of detected

344 glucosinolates in 268 wild *Brassica oleracea* plants from five counties in the UK

Glucosinolate group	Common name	Chemical name	Precursor ion (m/z)	Product ion (m/z)	Fragmentor voltage (V)	Collision energy (V)	Retention time (min)
Aliphatic	Gluconapoleiferin	4-Hydroxy-4-Pentenyl glucosinolate	402	97	100	19.1	8.2
Aliphatic	Progoitrin	(2R)-2-Hydroxy-3-butenyl glucosinolate	388	97	100	18.6	2.8
Aliphatic	Gluconapin	3-Butenyl glucosinolate	372	97	100	18.2	7.3
Aliphatic	Sinigrin	2-Propenyl glucosinolate	358	97	100	15	1.6
Aliphatic	Glucoiberberin	3-methylthiopropylglucosinolate	406	97	100	19.2	12.9
Aliphatic	Glucocapparin	Methylglucosinolate	332	97	100	16.6	3.2
Aliphatic	Glucocheiralin	3-Methylsulfonylpropyl	438	97	100	20.3	8.8
Indolic	Glucobrassicin	3-Indolymethyl glucosinolate	447	97	100	20.7	1.79
Indolic	Hydroxyglucobrassicin	4-hydroxyindol-3-ylmethylglucosinolate	463	97	100	11.3	9.7

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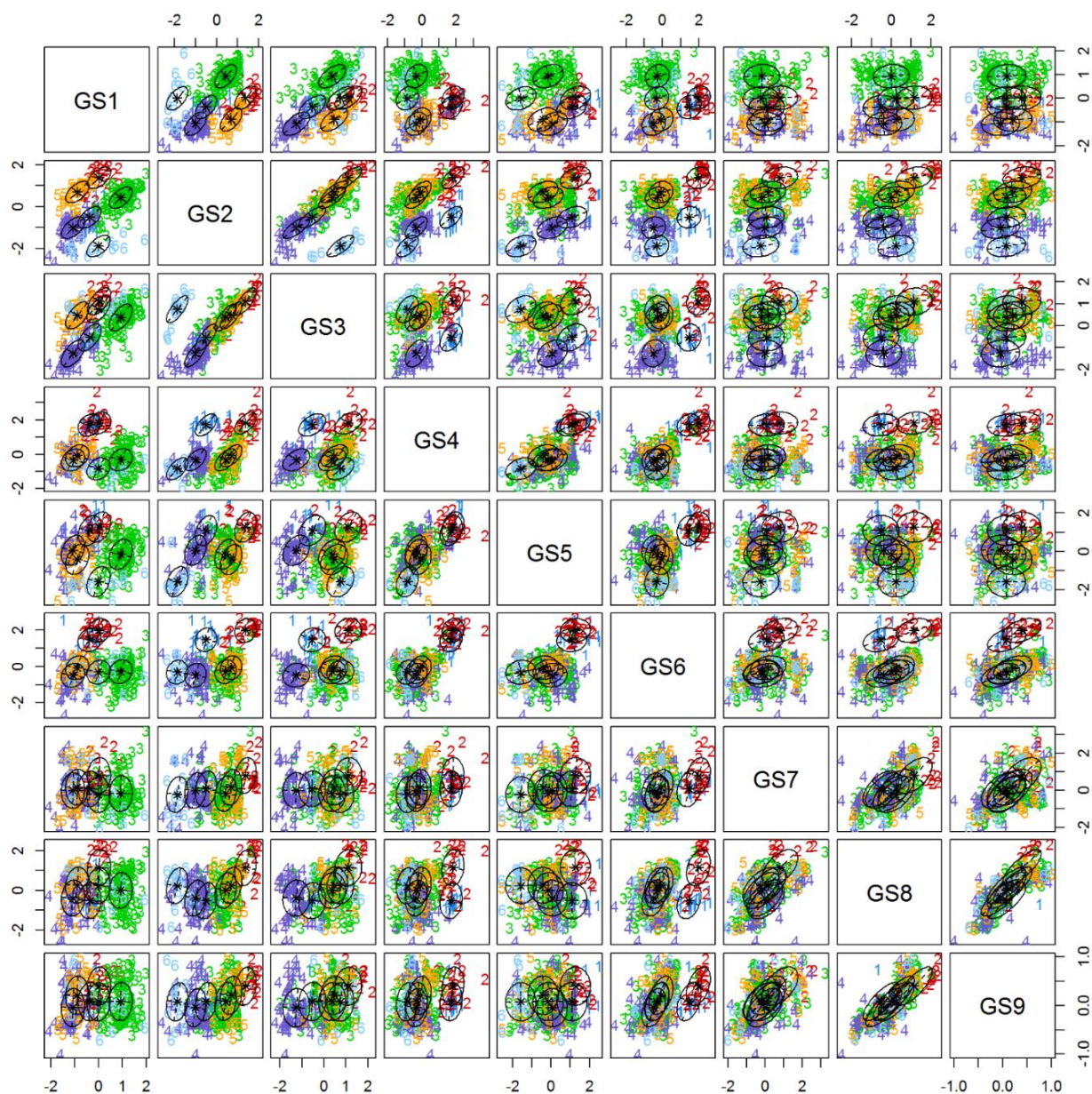
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348 *Clustering of wild plant glucosinolates reveals positive relationships.* Cluster analysis on the 268  
349 plants that contained all identified glucosinolates identified groups of chemically similar phenotypes.  
350 The best model according to BIC value was an equal-covariance model (EEE), with six clusters (BIC  
351 -5588.956). All six clusters tend to be consistent in their location in coordinate space (figure 2.3). For  
352 example, plants in cluster four exhibit low concentrations across compounds and plants in cluster  
353 two have generally high concentrations of all glucosinolates (figure 2.3).

354

355 Within each cluster, correlations between pairs of glucosinolates were revealed (figure 2.3). Simple  
356 linear relationships were observed between glucosinolates and their precursors (figure 2.4b:  
357 progoitrin and gluconapin; and figure 2.4d, glucobrassicin and hydroxyglucobrassicin), with the  
358 exception of sinigrin and glucoiberberin (figure 2.4c), which have an extra biosynthesis step between  
359 them (figure 2.1). Unexpectedly, concentrations of the aliphatic glucosinolate, glucocheirolin,  
360 appear to be highly correlated with concentrations of glucobrassicin; an indole glucosinolate from a  
361 different biosynthetic pathway (figure 2.2). There is also a positive association between aliphatic  
362 compounds sinigrin and progoitrin, representing the most derived glucosinolates from the 3-carbon  
363 and 4-carbon chains (figure 2.1), and three different intercepts emerge depending on cluster: for  
364 example, plants in clusters two and five are further separated by a step change in concentration of  
365 progoitrin (figure 2.4c). This pattern of separation is also seen between progoitrin and gluconapin,  
366 where plants in cluster six are differentiated by increased gluconapin concentrations.

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Figure 2.3. Scatterplot matrix of wild *Brassica oleracea* plants from five counties in the UK with standardized glucosinolate concentration on the X and Y axis. Plants are classified by similarities in glucosinolate concentrations into a six component, equal-covariance model (EEE). Ellipses superimposed onto the plot correspond to covariances of components or cluster. Aliphatic compounds = GS1: sinigrin, GS2: progoitrin, GS3: gluconapin, GS4: gluconapoleiferin, GS5: glucoiberberin, GS6: glucocapparin, GS7: glucocheirolin. Indole glucosinolates = GS8: glucobrassicin, GS9: hydroxyglucobrassicin. Patterns of positive correlations are observed between many compounds, both within and between biochemical pathways.

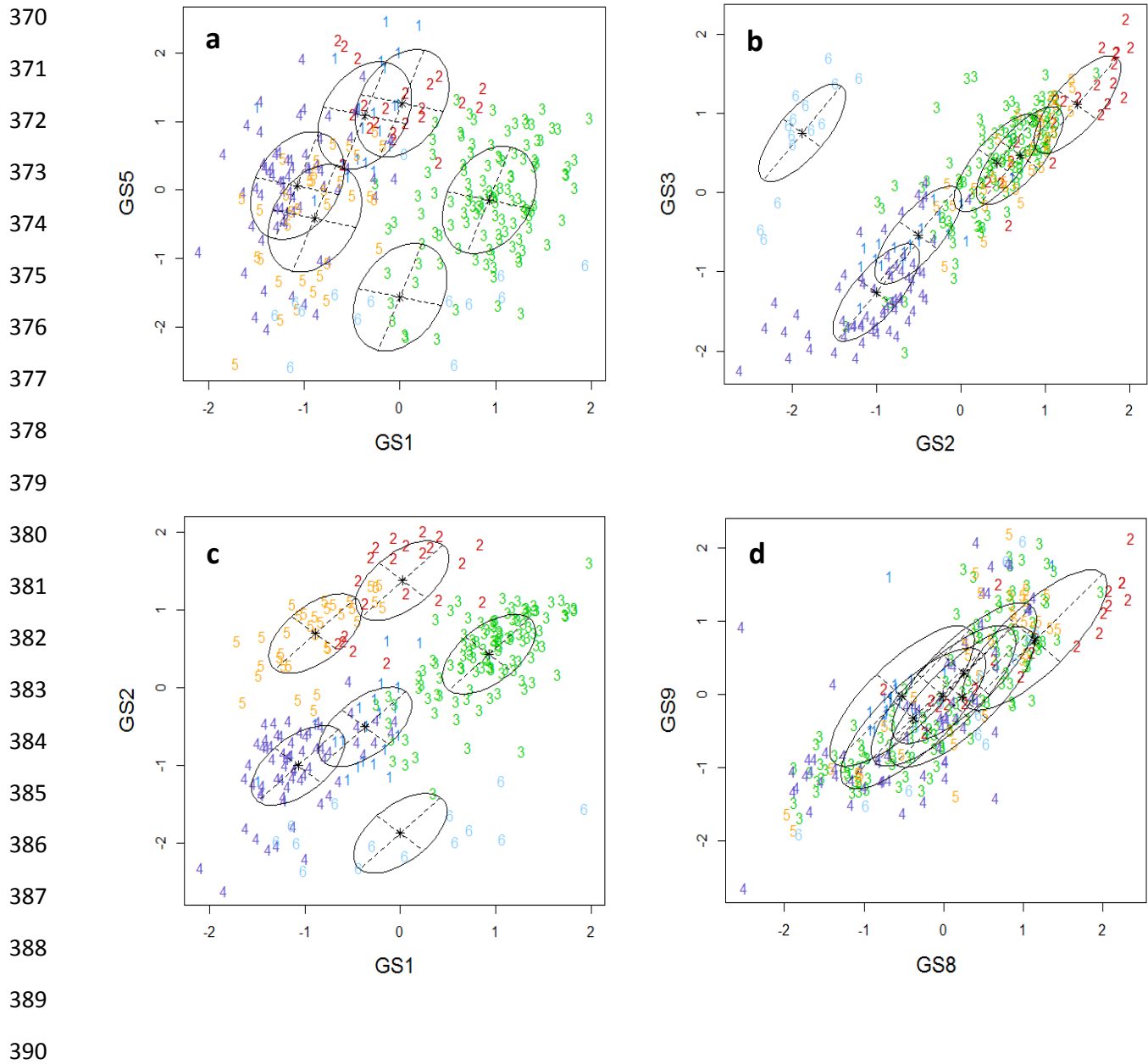


Figure 2.4. Pair plots for glucosinolate variables which exhibit associations in figure 3.3, with standardized glucosinolate concentration on the X and Y axis. Selected glucosinolates are: GS1 (sinigrin), GS2 (progoitrin), GS3 (gluconapin), GS5 (glucoiberverin), GS8 (glucobrassicin) and GS9 (hydroxyglucobrassicin). Positive linear relationships are observed between all pairs of these glucosinolates within and across the six clusters. Further separation of plants is revealed in panel c, with clusters two and five expressing high concentrations of progoitrin, and in panel b where plants in cluster six have high concentrations of gluconapin.

391

392 *Patterns of glucosinolate clustering in common garden plants is similar to that of wild plants.*  
393 In order to test whether patterns of glucosinolate variation observed for wild plants were purely  
394 environmental, we performed model-based clustering on plants reared under uniform conditions in a  
395 common garden. As for the analysis of wild plants, the best model according to BIC value was an  
396 EEE model with six clusters (BIC -7257.163). Cluster analysis on the 360 plants that contained all  
397 identified glucosinolates identified groups of glucosinolate concentrations, with relationships similar  
398 to those found for wild plants (figure 2.5). For example, positive correlations still persist for  
399 glucosinolates and their precursors (figure 2.6, b & d), and between compounds from different  
400 pathways (figure 2.5). However, the strength of correlations are weaker compared to those found  
401 between glucosinolates in wild plants and there are further differences in cluster separation. For  
402 example, whilst the positive association between sinigrin and progoitrin is still evident, there is now  
403 further cluster separation by a change in concentration of progoitrin (figure 2.6c). Additionally, for  
404 the relationship between progoitrin and gluconapin, which for wild plants exhibited separation of one  
405 cluster on gluconapin levels, we now see two clusters differentiating from the other groups on this  
406 axis.

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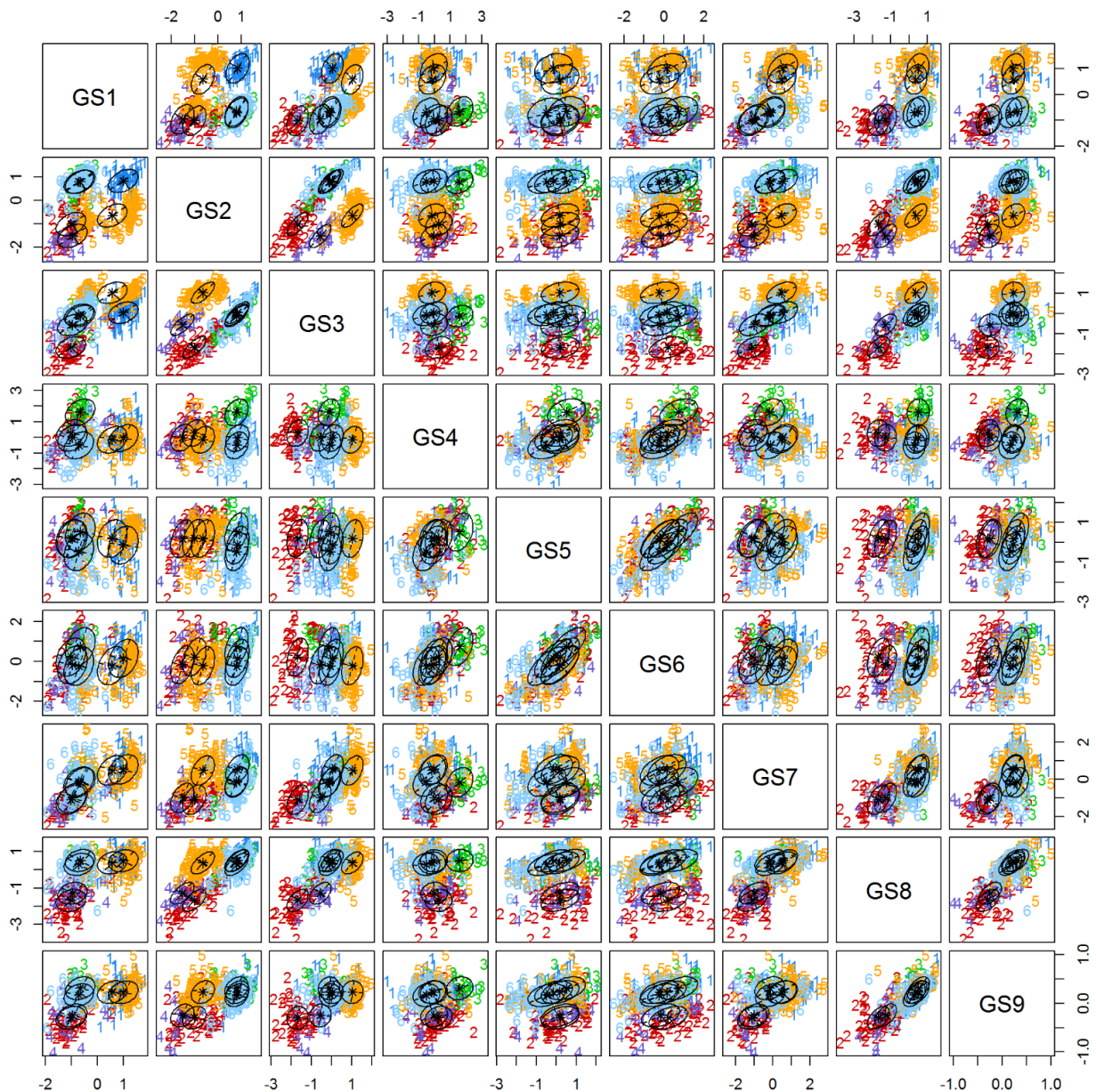
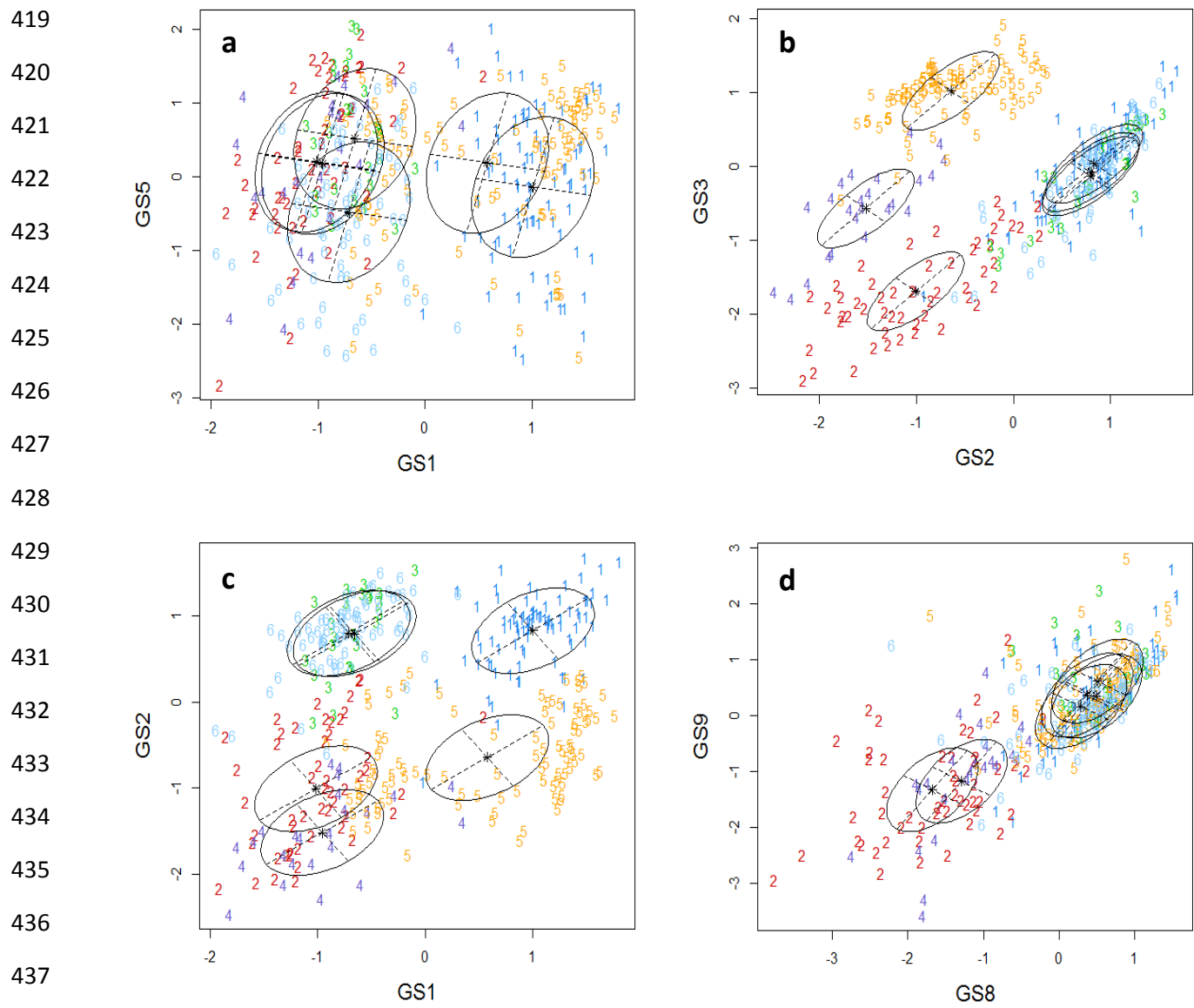


Figure 2.5. Scatterplot matrix of common garden reared *Brassica oleracea* plants from three counties in the UK, with standardized glucosinolate concentration on the X and Y axis. Plants are classified by similarities in glucosinolate concentrations into a six component, equal-covariance model (EEE). Ellipses superimposed onto the plot correspond to covariances of components or clusters. Aliphatic compounds = GS1: sinigrin, GS2: progoitrin, GS3: gluconapin, GS4: gluconapoleiferin, GS5: glucoiberberin, GS6: glucocapparin, GS7: glucocheirolin. Indole glucosinolates = GS8: glucobrassicin, GS9: hydroxyglucobrassicin. Patterns of positive associations and cluster differentiation are extremely similar to those found for the analysis of wild plants.



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 439 Figure 2.6. Pair plots for glucosinolate variables which exhibit associations in figure 3.5, with standardized  
 440 glucosinolate concentration on the X and Y axis. Selected glucosinolates are: GS1 (sinigrin), GS2 (progoitrin),  
 441 GS3 (gluconapin), GS5 (glucoiberverin), GS8 (glucobrassicin) and GS9 (hydroxyglucobrassicin). Patterns of  
 442 positive linear relationships and separation between glucosinolate clusters observed are similar to those found for  
 443 the analysis of wild plants. Clusters in the relationship between sinigrin and progoitrin and more spread, but two  
 444 intercepts can still be seen. However for common garden plants, an additional split is seen between clusters  
 differentiating gluconapin and progoitrin. Two clusters are now separated by increasing gluconapin  
 concentrations.

445 *Importance of sinigrin, progoitrin and gluconapin for wild and common garden cluster*  
446 *classification.* In order to identify glucosinolate features which may be of particular importance, we  
447 performed dimension reduction by reducing clusters through a linear set of combinations, or  
448 directions, of the original glucosinolate variables. For wild plants, the first two directions (linear  
449 combinations of original glucosinolate concentration means) account for most of the clustering  
450 structure (table 2.2). Changes in mean concentrations of the aliphatic glucosinolates, sinigrin and  
451 progoitrin, contribute strongly in separating plants into six clusters (table 2.2, figure 2.7a). Plants in  
452 clusters one to five appear to separate according to Direction 1, which is heavily weighted by  
453 negative contributions of sinigrin and progoitrin (table 2.2), whereas plants in cluster six split from  
454 the other clusters by Direction 2, which has a positive contribution from sinigrin and negative values  
455 of progoitrin (table 2.2, figure 2.7a). This suggests most of the clustering information is derived from  
456 changes in the concentrations of these two compounds.

457  
458 For common garden plants, the first two directions (linear combinations of original glucosinolate  
459 concentration means) also accounted for the clustering structure (table 2.2). However, the first  
460 direction is little influenced by sinigrin and is heavily weighted by positive contributions of  
461 progoitrin and negative contributions from its precursor, gluconapin. For the second direction, this  
462 relationship changes and gluconapin contributes little, with negative values of sinigrin and progoitrin  
463 influencing the clustering structure. Changes in sinigrin, gluconapin and progoitrin contribute  
464 strongly in separating plants into six clusters (figure 2.7b). Plants in clusters one, two and six  
465 separate according to Direction 2, which is weighted by negative sinigrin and progoitrin (table 2.2).  
466 Clusters four and five also separate by Direction 2, but are additionally differentiated from other  
467 clusters by Direction 1, which is weighted by positive progoitrin and negative gluconapin.

468 Therefore, whilst wild and common garden plants share the important feature of being differentiated  
469 according to changing values of sinigrin and progoitrin, there are key differences: wild plants  
470 chemotypes are dissociated by sinigrin versus progoitrin whilst common garden plants are further  
471 dissociated by progoitrin versus its precursor, gluconapin.

472

473 *Geography does not fully explain patterning of wild plant glucosinolate clusters.* The frequency of  
474 wild plants in each glucosinolate cluster varied significantly between counties ( $\chi^2_{20} = 185.74$ ,  
475  $P < 0.001$ ). However, county did not fully delimit the six clusters (figure 2.8): each of the six  
476 glucosinolate clusters did not contain plants from a single region but contained individual plants  
477 from all counties, with the exception of clusters one and two, which are restricted to plants from  
478 Cornwall (figure 2.8).

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Table 2.2. Estimated basis vectors (linear combinations of original glucosinolate features)

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spanning the reduced subspace given by dimension reduction for model-based clustering in

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wild and common garden plants. Importance is quantified by associated eigenvalues.

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Glucosinolate	Linear combinations of glucosinolates for wild plants		Linear combinations of glucosinolates for common garden plants	
	Direction 1	Direction 2	Direction 1	Direction 2
<b>Sinigrin</b>	<b>-0.725</b>	<b>0.681</b>	-0.163	<b>-0.417</b>
<b>Progoitrin</b>	<b>-0.420</b>	<b>-0.563</b>	<b>0.768</b>	<b>-0.660</b>
<b>Gluconapin</b>	0.182	-0.089	<b>-0.596</b>	-0.078
<b>Gluconapoleiferin</b>	0.330	0.237	-0.001	0.140
<b>Glucoiberberin</b>	0.090	-0.014	-0.043	0.275
<b>Glucocapparin</b>	0.313	-0.0135	0.069	0.219
<b>Glucocheirolin</b>	0.192	0.0546	0.031	-0.069
<b>Glucobrassicin</b>	-0.070	-0.332	-0.121	-0.425
<b>Hydroxyglucobrassicin</b>	-0.071	-0.199	-0.071	-0.231
<b>Eigenvalues</b>	0.748	0.671	0.839	0.634
<b>Cumulative %</b>	34.808	66.051	44.904	78.861

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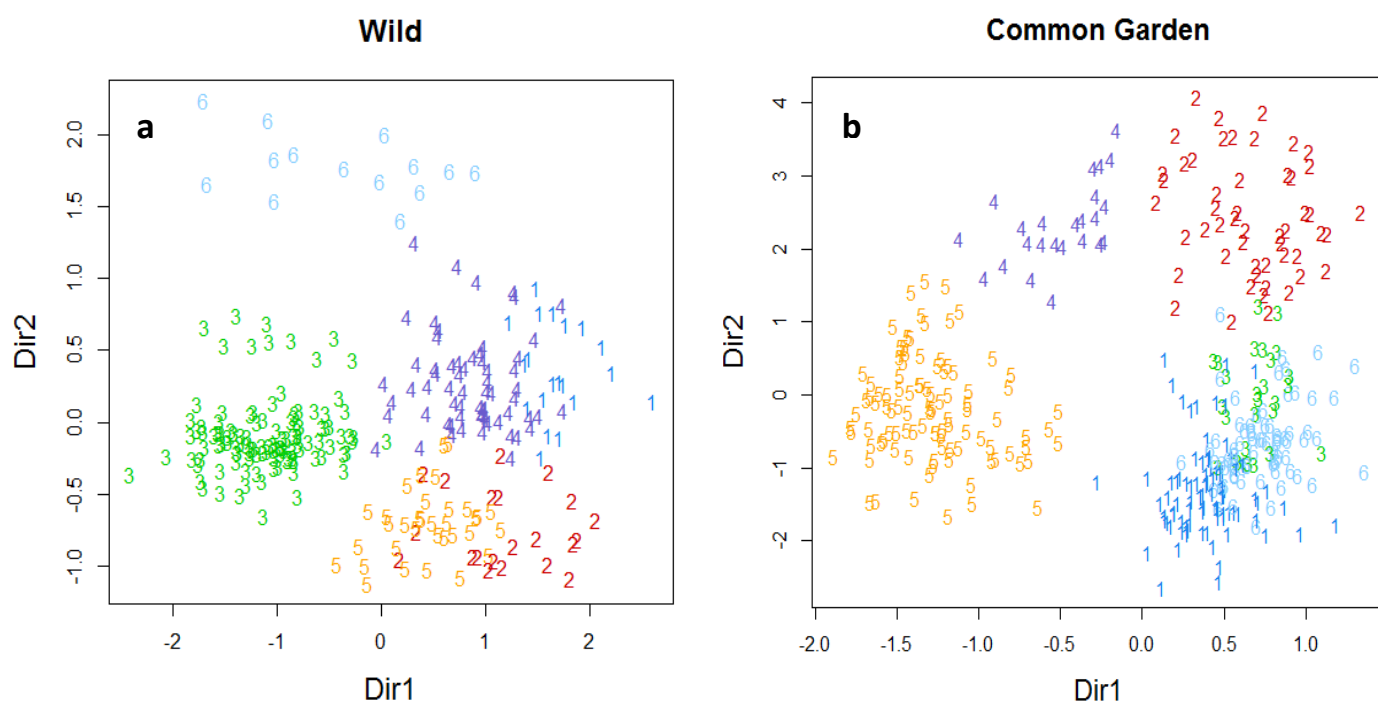


Figure 2.7. Scatterplot of clustering structure in reduced glucosinolate subspace. Direction 1 (on the x axes) and Direction 2 (on the y axes) are linear combinations of nine identified glucosinolates in *Brassica oleracea* plants from wild populations in five counties, and common garden plants from four counties. For wild plants (a), glucosinolate clusters 1- 5 appear to separate according to Direction 1, which is heavily weighted by negative contributions of sinigrin and progoitrin (table 2.2), whereas plants in cluster 6 split from the other clusters by Direction 2, which has a positive contribution from sinigrin and negative values of progoitrin. For common garden plants (b), clusters 1, 2 and 6 separate according to Direction 2, which is weighted by negative sinigrin and progoitrin (table 2.2). Clusters 4 and 5 also separate by Direction 2, but are additionally differentiated from other clusters by Direction 1, which is weighted by positive progoitrin and negative gluconapin (table 2.2).

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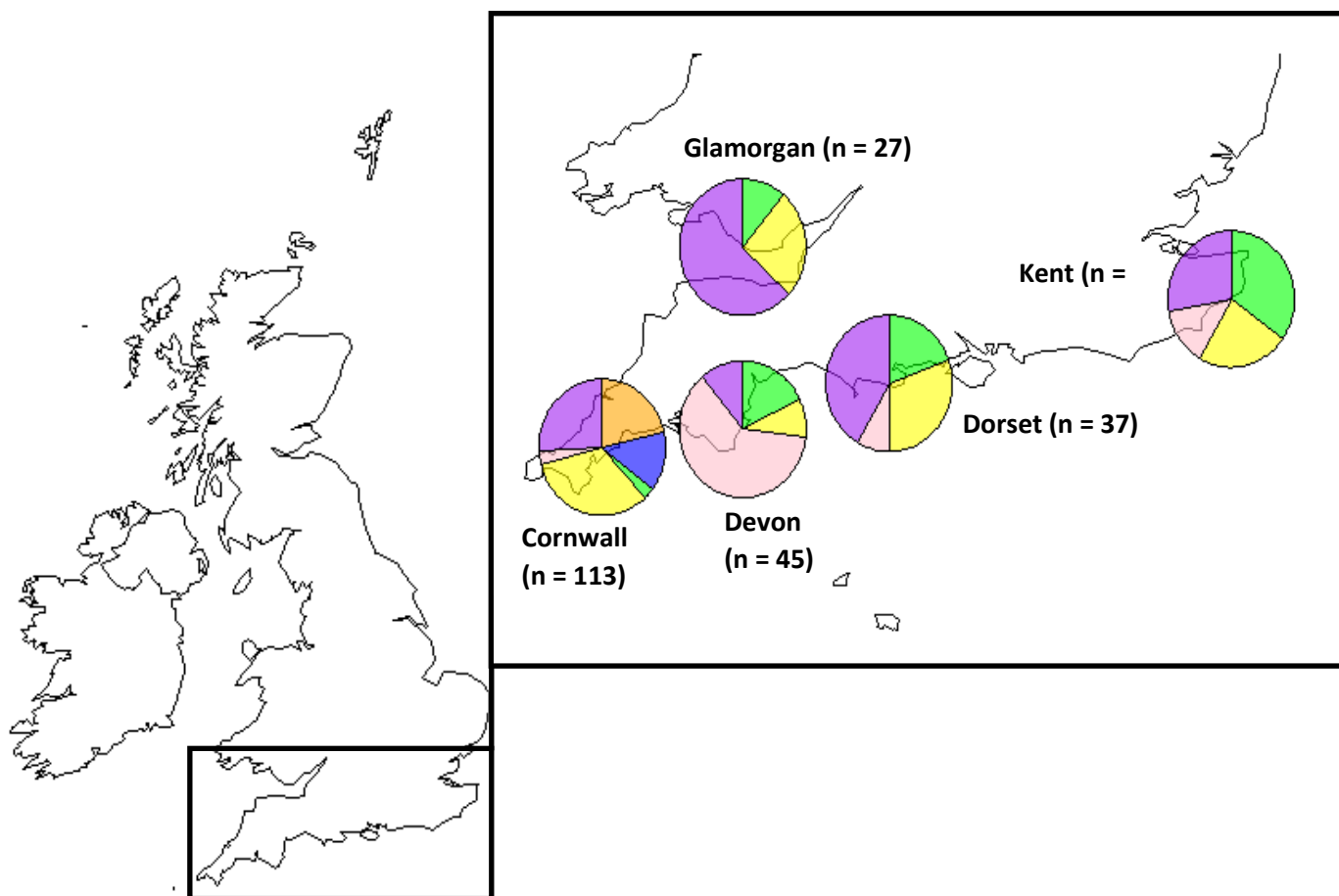


Figure 2.8. Frequency of plants in glucosinolate clusters for each of the five counties in the UK. Cluster 1 = orange; cluster 2 = blue; cluster 3 = green; cluster 4 = yellow ; cluster 5 = pink ; cluster 6 = purple. Number of plants sampled per county are given in parantheses. The presence of “rare” phenotypes (clusters one and two) are observed only in Cornwall.

## DISCUSSION

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533

534 Describing intraspecific variation, and the associations between different components of defence  
535 phenotypes, is an important step towards defining selection pressures underlying secondary  
536 metabolite diversity (Agrawal et al. 1998). In this study, we performed model-based cluster analysis  
537 to study the variation of multiple traits simultaneously and identify homogenous groups, predicting  
538 that, according to classic plant defence theory; negative phenotypic correlations would be revealed  
539 between constitutive and inducible glucosinolates, and between compounds from the same  
540 biosynthetic pathway sharing the same precursor. Each cluster described a multi-trait glucosinolate  
541 phenotype occurring at varying frequencies in the counties sampled. We found no evidence of trade-  
542 offs between glucosinolates, instead positive associations within and between glucosinolate  
543 structural groups were observed. These patterns were consistent for both wild and common garden  
544 plants, suggesting that associations are not due to clinal changes in resource allocation, and  
545 furthermore, simultaneous production of multiple defences is not constrained. These results add to a  
546 growing body of evidence reporting a lack of trade-offs between different plant defences, suggesting  
547 other mechanisms may be maintaining variation in secondary metabolites profiles.

548

549 *Biochemical patterns of glucosinolate variation in wild and common garden plants reveal positive*  
550 *correlations*

551 The majority of theories regarding patterns of defence allocation in plants predict the occurrence of  
552 trade-offs between constitutive and inducible defences due to physiological and/or ecological costs  
553 (Rhoades 1979; Mattson et al 1988; Karban and Myers 1989). We therefore predicted negative  
554 correlations between concentrations of compounds belonging to different groups within a class, or  
555 between different classes of chemical compounds. However, we found no negative associations, and



556 therefore no evidence to support the prediction of a trade-off between constitutive and inducible  
557 defences. Indeed, our results indicate that positive associations between glucosinolates may be  
558 widespread. Simple linear relationships were observed between glucosinolates and their precursors;  
559 however we also found positive correlations between glucosinolates from different biosynthetic  
560 pathways and structural groups. For example, an aliphatic compound, glucocheirolin is highly  
561 correlated with concentrations of the indole glucosinolate glucobrassicin. In addition, we found a  
562 positive association between the aliphatic compounds sinigrin and progoitrin, representing the most  
563 derived glucosinolates from the 3-carbon and 4-carbon chains (figure 2.1). These patterns of  
564 association were also found for plants grown under common environmental conditions, suggesting  
565 they are not simply reflecting clinal variation in resource availability. However, the strength of  
566 correlations between different glucosinolates for common garden plants was weaker. The most likely  
567 explanation is that common gardens remove the influence of environmental covariates, such as soil  
568 sulphur and nitrogen or wind exposure.

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570 Positive correlations between two compounds may indicate linkage of genes or linkage of  
571 biosynthetic reaction sequences (Zangerl 1970; White 1983). For example, our finding of co-  
572 expression of glucosinolates sharing the same precursor suggests that for these plants, the amount of  
573 the precursor is not limited (Koricheva et al. 2004). Our finding of positive associations between  
574 inducible indole compounds and constitutive aliphatic glucosinolates contradicts the findings of a  
575 recent meta-analysis investigating the covariance of different defensive plants traits. Significant  
576 negative correlations were reported between constitutive and inducible defences (Koricheva et al.  
577 2004). However, in their study of 31 datasets from a range of plant species, they found variation in  
578 constitutive defences explained a low percentage of the variance observed in inducible responses,  
579 suggesting environmental variation may be more important in determining inducible chemical levels

580 (Koricheva et al. 2004). Furthermore, a study on glucosinolate expression in *Brassica nigra* found a  
581 strong positive correlation between aliphatic sinigrin and the indole glucosinolate, glucobrassicin in  
582 greenhouse experiments (Traw 2002).

583

584 Results from studies of other brassica species have found either no correlation between aliphatic and  
585 indole glucosinolates in *B. rapa*, (Cipollini et al. 2003), a negative correlation between a single  
586 compound and intra- and interspecific competitive ability in *B. nigra* (Lankau 2008), or a negative  
587 correlation between trichome density and total glucosinolate concentration in *Arabidopsis thaliana*  
588 (Mauricio 1998). One explanation for the fact that our results differ from those previously reported,  
589 may be due to the differences in life histories of the species being considered: the above studies are  
590 of annual Brassica species, whereas wild cabbage is a long-lived plant with a life span of up to 20  
591 years. These are not, therefore, ecologically equivalent species, and the nature of selection operating  
592 on their glucosinolate profiles might differ. It must also be noted that in this study we did not  
593 measure growth or fitness parameters, and so cannot know if the positive correlations between  
594 different defences reported here exacted physiological costs for plants.

595

596 In this study we were also unable to examine the ecological implications of associations between  
597 glucosinolate defences. It may be that in certain contexts plants may produce several types of  
598 defence without paying considerable trade-offs. The benefits of possessing a functional multiplicity  
599 of defensive traits may outweigh costs by protecting against a broader range of herbivores and  
600 pathogens than an individual mechanism (eg, Langenheim 1994; Romeo 1998; Close and McArthur  
601 2002); or by acting synergistically to provide efficient defence against specialist herbivores, as has  
602 been reported for several plant toxins (Berenbaum and Neal 1985; Challis and Hopwood 2003;  
603 Richards et al. 2012). Under this scenario, there are no genetic constraints on the evolution of

604 multiple glucosinolate defensive traits, and selection may favour the maintenance of multiple  
605 defences because it increases the probability of possessing a trait that confers resistance against  
606 organisms with which the plant interacts (Koricheva et al. 2004). In order to investigate whether the  
607 chemical associations we observed are adaptive, future experiments are recommended which  
608 manipulate plant ability to express high concentrations of multiple compounds, and measure the  
609 effects of this on herbivore abundance and performance, in addition to plant fitness.

610

611 *Glucosinolate phenotypes are separated by changing concentrations of particular compounds*

612 All plants in our study produced sinigrin, progoitrin and gluconapoleiferin, indicating all plants  
613 possess functional alleles at the *GS-ELONG*, *GS-ALK* and *GS-AOP-OH* loci (figure 2.1). This result  
614 contradicts previous findings which have found variation in the expression of these compounds. For  
615 example, Newton et al. (2009) reported that plants from wild *B. oleracea* populations in the South  
616 West were highly variable in aliphatic glucosinolate profiles, variation that was driven by presence  
617 or absence of sinigrin and progoitrin, suggesting genetic polymorphism for these compounds. The  
618 differences in our findings may be due to improved detection capabilities of HPLC-MS (Mohn et al.  
619 2007). Whilst we did not find polymorphism in the presence of these compounds, our results do  
620 suggest that changing concentrations of certain aliphatic glucosinolates is a key driver separating  
621 plant glucosinolate phenotypes: plants in clusters were separated by a step change in progoitrin  
622 (figure 2.4c), and dimension reduction revealed that changes in sinigrin and progoitrin were driving  
623 glucosinolate clustering. Thus, as in Newton et al. (2009) our findings still indicate polymorphism  
624 for these key metabolites.

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627 However, the dominant discriminators for wild and common garden plant chemotypes change in  
628 importance, and also in the contribution of the different glucosinolates. For example, common  
629 garden plant chemotypes were further dissociated by a negative relationship between progoitrin and  
630 its chemical precursor, gluconapin, indicating that there could be a trade-off between these two  
631 compounds caused by a single functional allele (figure 2.1). If a positive correlation between these  
632 two compounds is beneficial in the wild, such that it overcomes any associated costs, it may be that a  
633 trade-off is only revealed in a common garden environment where plants are free of many of the  
634 selection pressures operating in wild populations. However, in this study we have made the  
635 assumption that our use of a common garden design removes variation in the resources available to  
636 plants. In actuality, spatial heterogeneity in soil resources within such an environment may still exist.  
637 For example, available sulphur may have been limited, and previous work has shown that sulphur-  
638 deficient plants downregulate glucosinolate biosynthesis, in particular, aliphatic compounds (Rosen  
639 et al 2005).

640

641 *Patterns of glucosinolate variation in the wild were not fully explained by geography*

642 Whilst there were significant regional differences, location did not fully explain the six glucosinolate  
643 clusters: each of the six glucosinolate clusters did not contain plants from a single region, but  
644 contained individual plants from most or all counties. It is possible that variation in heritable  
645 glucosinolate profiles may have arisen by chance through a combination of founder effects and  
646 genetic drift (Mithen et al. 1995b). However, large differences in allele frequencies occur at loci  
647 which regulate the occurrence of particular glucosinolates, in contrast to alleles at isozyme loci,  
648 suggesting variation is the result of differential selection occurring in different regions (Mithen et al.  
649 1995b).

650

651 Glucosinolates have been shown to have differential effects on insect herbivore responses, generally  
652 acting as deterrents for generalist feeders, but serving as host-acceptance cues and feeding stimulants  
653 for specialist species (Jaenike 1990; Renwick 1994; Smallegange et al. 2007a). Therefore differential  
654 selection driven by variable herbivore preferences is a potential ecological mechanism for  
655 maintaining diversity in secondary metabolite concentration and composition within this plant  
656 species (Dethier 1954; Cornell and Hawkins 2003b). Our results suggests that there may be selection  
657 for plant chemotypes which vary in concentrations of key metabolites, sinigrin and progoitrin, and  
658 future work will aim to investigate the significance of these compounds in determining patterns of  
659 host plant utilization by herbivores in these populations.

660

661 In summary, we have described multi-trait glucosinolate chemotypes which vary in their regional  
662 frequencies, and we have revealed positive associations among and within glucosinolate groups,  
663 indicating that investment in multiple defences may not be costly for wild plants. However, the  
664 strength of glucosinolate correlations for common garden plants was weaker, and there is evidence of  
665 a potential trade-off between progoitrin and its precursor, indicating that patterns of co-expression  
666 may change rapidly in novel environments. Our study adds to a growing body of evidence that finds  
667 little support for trade-offs constraining the evolution of multiple defences in plants, and we suggest  
668 compounds may be interacting synergistically, or that variation in particular compounds may be  
669 directed against different sets of herbivores (Mauricio 1998). We therefore recommend future studies  
670 of this system investigate the significance of these results in interactions with naturally occurring  
671 herbivore species.

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### CHAPTER THREE

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677

HERBIVORE RESPONSES TO INTRASPECIFIC VARIATION IN PLANT SECONDARY

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METABOLITES IN WILD POPULATIONS OF *BRASSICA OLERACEA*

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## ABSTRACT

Intraspecific variation in plant secondary metabolite profiles is thought to be maintained by the fluctuating selection pressures imposed by variable preferences and aversions of herbivorous species. However, there remains a lack of consensus regarding the patterns of selection exerted by insect herbivores on plant defences, and our understanding of these processes has been limited by failure to measure whole herbivore assemblages, and their responses to complex plant chemotypes, in ecologically relevant settings. Here we investigate the responses of specialist and generalist herbivores to a wide range of glucosinolate secondary metabolites, across natural populations of *Brassica oleracea*. Using targeted high-performance liquid chromatography- mass spectrometry, we identified ten compounds from two glucosinolate chemical groups, and demonstrated a relationship between the differential contribution of each chemical to plant phenotype and host utilization by specialist and generalist herbivores. We show that herbivore responses to glucosinolate phenotype vary across herbivore species, regardless of feeding category, but we also show that all specialist herbivores share a preference for the aliphatic glucosinolate progoitrin. Our results suggest that fluctuating selection for secondary metabolite profiles, driven by variation in preference across herbivore species, could help to maintain inter- and intra-population variation in glucosinolate concentration and composition in wild brassica species. We also propose a direct link between concentrations of progoitrin and the prevalence and intensity of herbivory by brassica specialists.

718

## INTRODUCTION

719 The process of coevolution is widely accepted as a paradigm for the evolution of the extraordinary  
720 taxonomic diversity in plant secondary metabolites, and for the adaptive radiation of phytophagous  
721 herbivore lineages (Fraenkel 1959; Ehrlich and Raven 1964; Berenbaum and Zangerl 2008). At a  
722 micro-evolutionary scale, the coevolutionary framework hypothesizes that high intraspecific  
723 variation in secondary metabolite composition and concentration is maintained through co-evolved  
724 chemical preferences and aversions of herbivorous species that vary in their prevalence in plant  
725 populations (Dethier 1954; Cornell and Hawkins 2003a). Specifically, it is predicted that generalist  
726 herbivores are effectively deterred, or negatively affected by plant secondary compounds.

727 Conversely, specialist species with narrower diet breadths and physiological and/or behavioral  
728 counter-adaptations, may be attracted to oviposit or feed by secondary chemicals characteristic to  
729 their host plants (Van de Meijden 1996). Thus for a plant, there may exist an ecological trade-off  
730 between defending against generalist species and attracting specialist attackers (Van de Meijden  
731 1996). If the ratio of specialist to generalist herbivores within a plant population varies in space and  
732 time, the resulting fluctuating selection pressures may maintain genetic variation in secondary  
733 metabolite profiles (Giamoustaris and Mithen 1995; Strauss et al. 2002; Lankau 2007).

734

735 However, this theory has been criticized on a number of points (Jermy 1993; Janz 2011). For  
736 example, whilst it is generally accepted that secondary metabolites play an important role in plant  
737 defence (Bennett and Wallsgrave 1994), it has been argued that selection on plants by herbivores  
738 may be too weak and variable to drive evolutionary change (Jermy 1993). In addition, conflicting  
739 selection pressures from multiple herbivore species may result in diffuse, rather than pairwise  
740 coevolution (Janzen 1980). Therefore there remains a lack of consensus regarding the patterns of  
741 selection exerted by insect herbivores on plant secondary profiles. Three key problems have



742 hampered development of the necessary evidence base: first, plant-insect interactions tend to be  
743 studied in controlled conditions using experimental approaches that oversimplify the complexity  
744 inherent in natural systems; second, secondary metabolites are often examined on a case-by-case  
745 basis, i.e. studying single compounds; and third, herbivore species responses to compounds are often  
746 studied in isolation. Our goal here is to address all three of these issues using surveys of herbivore  
747 responses to complex secondary profiles in natural plant populations.

748

749 Our study system, the wild cabbage *Brassica oleracea*, provides an excellent example of a plant  
750 species with complex secondary metabolite profiles, attacked by a wide range of generalist and  
751 specialist herbivore species. The glucosinolates produced by Brassicaceae are sulfur- and nitrogen-  
752 containing secondary compounds (Fahey et al. 2001) that have long been recognized as plant  
753 defences due to their degradation upon tissue damage into various biological active products (Bones  
754 and Rossiter 1996; Fahey et al. 2001; Raybould and Moyes 2001). These highly variable compounds  
755 can be classified according to their amino acid precursors, and the type of modification to a variable  
756 side-chain, into aliphatic, aromatic or indolic groups (Halkier and Gershenzon 2006). High  
757 concentrations of these compounds have been shown to provide effective defence against some  
758 generalist herbivores (Hopkins et al. 2009; Müller et al. 2010), yet conversely increase attraction to  
759 specialist insect species (Nielsen 1978; Agrawal and Kurashige 2003; Müller et al. 2010).

760 Furthermore, polymorphism in the expression of aliphatic glucosinolates compounds, in particular  
761 sinigrin and progoitrin, have been linked to attraction and aversion by specialist and generalist  
762 herbivores (Lankau 2007; Newton et al. 2009b). It is therefore plausible that the significant inter-and  
763 intra-population variation in glucosinolate concentration and composition observed in wild brassica  
764 species could be maintained by differential attack from fluctuating populations of specialists and  
765 generalists species (Giamoustaris and Mithen 1995; Lankau 2007).

766 However, a survey of the literature describing herbivore responses to individual glucosinolates  
767 revealed substantial variation in the direction of response, with positive, negative and equivocal  
768 responses often found for the same species (table 3.1). Furthermore, a recent study investigating  
769 patterns of host utilization across wild populations of *B. oleracea* in response to qualitative variation  
770 in sinigrin and progoitrin, found a positive association between snails, a generalist herbivore, and  
771 sinigrin (Newton et al. 2010): this result hardly fits the prediction that generalists should avoid  
772 defence metabolites. The same authors also reported a positive relationship between sinigrin and the  
773 presence of the brassica specialist butterfly *Pieris brassicae* (Newton et al. 2009b), and therefore  
774 concluded that herbivores showing similar responses to glucosinolates could not be grouped into  
775 generalists and specialists.

776

777 The inconsistencies in reported herbivore responses to glucosinolates may be due to the tendency to  
778 consider plant-herbivore interactions with single compounds. In reality, herbivores are confronted  
779 with differences in relative concentrations of a suite of compounds from different structural groups,  
780 resulting in complex plant chemotypes determined by both genetic and environmental effects. For  
781 example, aliphatic glucosinolate type and concentration are under strong genetic control and are  
782 highly heritable (Kliebenstein et al. 2001b), resulting in variable, yet relatively predictable  
783 phenotypes within and among populations. However, indole glucosinolates are highly plastic and  
784 may be rapidly induced in response to abiotic and biotic stressors in a plant's local environment  
785 (Agrawal et al. 2002). In order to fully understand the significance of plant secondary metabolites in  
786 determining patterns of host use, we need to study herbivore responses to variability across the full  
787 diversity of the chemical phenotype, and other potentially important functional plant defence traits,  
788 in natural communities. Current methods in chemical analyses with highly sensitive detection

789 capabilities now allow rapid, quantitative assessment of plant metabolite profiles, yielding new  
790 potential for studying herbivore responses across a suite of compounds in wild populations.

791

792 Here we use surveys of herbivore prevalence and glucosinolate profiles, in natural populations, to  
793 distinguish among four main patterns of plant-chemotype-herbivore-assemblage interaction. First,  
794 we recognize the possibility that herbivore species have no clear preference for, or aversion to, plant  
795 secondary metabolites in wild populations, suggesting that the observed diversity of secondary  
796 metabolites is neutral with respect to herbivory. The sheer weight of evidence from lab, greenhouse  
797 and ecological studies makes this an unlikely outcome. A second possible pattern is that all herbivore  
798 species show the same set of preferences for glucosinolate profiles. This also seems unlikely, given  
799 the known attractant/deterrence status of various glucosinolates. A third pattern is that each herbivore  
800 species has evolved its own set of preferences for, and aversions to, glucosinolate profiles. In this  
801 scenario, we hypothesize that each species will exhibit distinct associations with plant glucosinolate  
802 profiles, irrespective of feeding specialization. Fourth, if the whole system can be considered as  
803 being shaped by opposing selection pressures due to generalist and specialist herbivores, we predict  
804 shared preferences among specialists, shared preferences among generalists, but fundamental  
805 differences between these two guilds. For completeness, we also incorporate measures of physical  
806 plant traits which have been previously highlighted as important for insect herbivories, such as leaf  
807 colour (Hamilton and Brown 2001; Green et al. 2015) and plant size (Wiklund 1984). Specifically,  
808 we assessed variation in the composition of glucosinolate profiles in six natural populations of wild  
809 cabbage, *Brassica oleracea* and investigated whether the presence of specialist and generalist  
810 herbivore species was associated with this variation.

Table 3.1 Studies showing the effect of different glucosinolates on the behaviour and performance of a subset of specialist insect herbivores and one generalist. Studies were included if they investigated herbivore responses to individual, rather than total, glucosinolates

Insect	Glucosinolate compound <sup>a</sup>	Type of study <sup>b</sup>	Behaviour measured	Effect <sup>c</sup>	Plant	Ref
<i>Brevicoryne brassicae</i>	Gluconapoleiferin <sup>a</sup>	G	Intrinsic rate of increase	+	<i>Brassica spp. and cultivars</i>	1
	Progoitrin <sup>a</sup>	F, G	Abundance, intrinsic rate of increase	+, -, 0	<i>Brassica oleracea, Brassica spp. and cultivars</i>	2, 3, 4, 1
	Gluconapin <sup>a</sup>	F	Abundance	+, 0	<i>B.oleracea</i>	4, 2
	Sinigrin <sup>a</sup>	F, L, C.G	Intrinsic rate of increase, feeding intensity, abundance, pupal mass	+, -, 0	<i>Brassica spp. and cultivars, Brassica nigra, B.oleracea</i>	1, 5, 2, 6, 7, 8, 9, 3, 4
	Glucobrassicinapin <sup>a</sup>	G	Intrinsic rate of increase	+	<i>Brassica spp. and cultivars</i>	1
	Glucobrassicin <sup>i</sup>	F	Abundance	-, 0	<i>B.oleracea</i>	4, 2, 10
<i>Pieris rapae</i>	Progoitrin <sup>a</sup>	G, F	Development time, pupal mass, abundance	+, 0	<i>B.oleracea, Brassica cultivars</i>	2, 11, 12, 9
	Gluconapin <sup>a</sup>	G, F	Development time, pupal mass, abundance	0	<i>B.oleracea, Brassica cultivars</i>	2, 11, 12
	Sinigrin <sup>a</sup>	L,G, F	Oviposition, feeding, development time, pupal mass, abundance	+, 0	Leaf wash, <i>B. oleracea cultivars</i>	13, 14, 15, 2, 11, 12, 9
	Glucoscheirolin <sup>a</sup>	L	Oviposition	+	Leaf wash	16
	Glucobrassicin <sup>i</sup>	L, G	Oviposition, sensilli development time, pupal mass, abundance	+, 0	Leaf wash, <i>B. oleracea cultivars, B.oleracea,</i>	13, 14, 16, 2, 11, 12
	Hydroxyglucobrassicin <sup>i</sup>	G	Development time, pupal mass	0	<i>B.oleracea, Brassica cultivars</i>	11, 12

<i>Pieris brassicae</i>	Progoitrin <sup>a</sup>	F	Abundance	<b>0</b>	<i>B. oleracea</i>	3, 9
	Gluconapin <sup>a</sup>	F	Abundance	<b>0</b>	<i>B. oleracea</i>	2
	Sinigrin <sup>a</sup>	G, F	Abundance, growth rate	+, -, <b>0</b>	<i>Brassica nigra</i> , <i>B. oleracea</i>	2, 16, 9
	Glucobrassicin <sup>i</sup>	L, F	Oviposition	+, <b>0</b>	Leaf washes, <i>B. oleracea</i>	18, 2
	Hydroxyglucobrassicin <sup>i</sup>	G	Growth rate	+	<i>B. nigra</i>	17
Snails	Sinigrin <sup>a</sup>	F, C.G	Abundance	+, -, <b>0</b>	<i>B. oleracea</i> , <i>B. nigra</i>	6, 9, 3
<i>Myzus persicae</i>	Gluconapin <sup>a</sup>	G	Intrinsic rate of increase	-	20 Brassica accessions	1
	Glucobrassicin <sup>i</sup>	G	Intrinsic rate of increase	+	20 Brassica accessions	1
	Hydroxyglucobrassicin <sup>i</sup>	G	Fecundity	-	<i>Arabidopsis thaliana</i> and purified extracts	19

<sup>a</sup> abbreviations used: <sup>a</sup>: Aliphatic compounds; <sup>i</sup>: Indole compounds

<sup>b</sup> C.G: Common garden; G: Greenhouse; F: Field study; L: Laboratory

<sup>c</sup> +: positive correlation; -: negative correlation; **0**: no correlation

References: <sup>1</sup>Cole 1997; <sup>2</sup>Moyes et al. 2000; <sup>3</sup>Newton et al. 2010; <sup>4</sup>Goodey et al. 2015; <sup>5</sup>Gabrys and Tjallingii 2002; <sup>6</sup>Lankau 2007; <sup>7</sup>Lankau and Strauss 2007; <sup>8</sup>Pratt et al. 2008; <sup>9</sup>Newton et al. 2009; <sup>10</sup>Mewis et al. 2006; <sup>11</sup>Gols et al. 2008; <sup>12</sup>Poelman et al. 2009; <sup>13</sup>Renwick et al. 1992; <sup>14</sup>Huang and Renwick 1993; <sup>15</sup>Renwick and Lopez 1999; <sup>16</sup>Stadler et al. 1995; <sup>17</sup>Smallegange et al. 2007; <sup>18</sup>van Loon et al. 1992 <sup>19</sup>Kim and Jander 2007

## MATERIALS AND METHODS

*Plant and Herbivore Surveys.* Plant preferences were assessed in June 2013 across six wild populations of *B. oleracea* in Dorset, England (figure 3.2). These populations have been shown previously to vary substantially in glucosinolate profiles and herbivory (Mithen et al. 1995; Newton et al. 2009a). 20 randomly selected plants in each population were surveyed for the presence of herbivores. All herbivores were identified to species level where possible (Kirk and Grey 1992) and recorded along with physical plant traits and environmental parameters, namely plant size (as measured by rosette and basal stem diameter), leaf colour and exposure. Leaf colour (green or purple, assessed visually), leaf toughness (assessed by touch) and exposure were assessed on an ordinal scale and given a score between 1-5. For leaf colour, lower numbers represented predominantly green leaved plants, with values increasing with purpleness. Leaf toughness likewise increased with scale and in the exposure assessment, plants in sheltered positions were given a score of one, and value increased with degree of exposure. Leaf tissue samples (1cm<sup>2</sup> sections excised from five randomly selected leaves) were harvested from all surveyed plants.

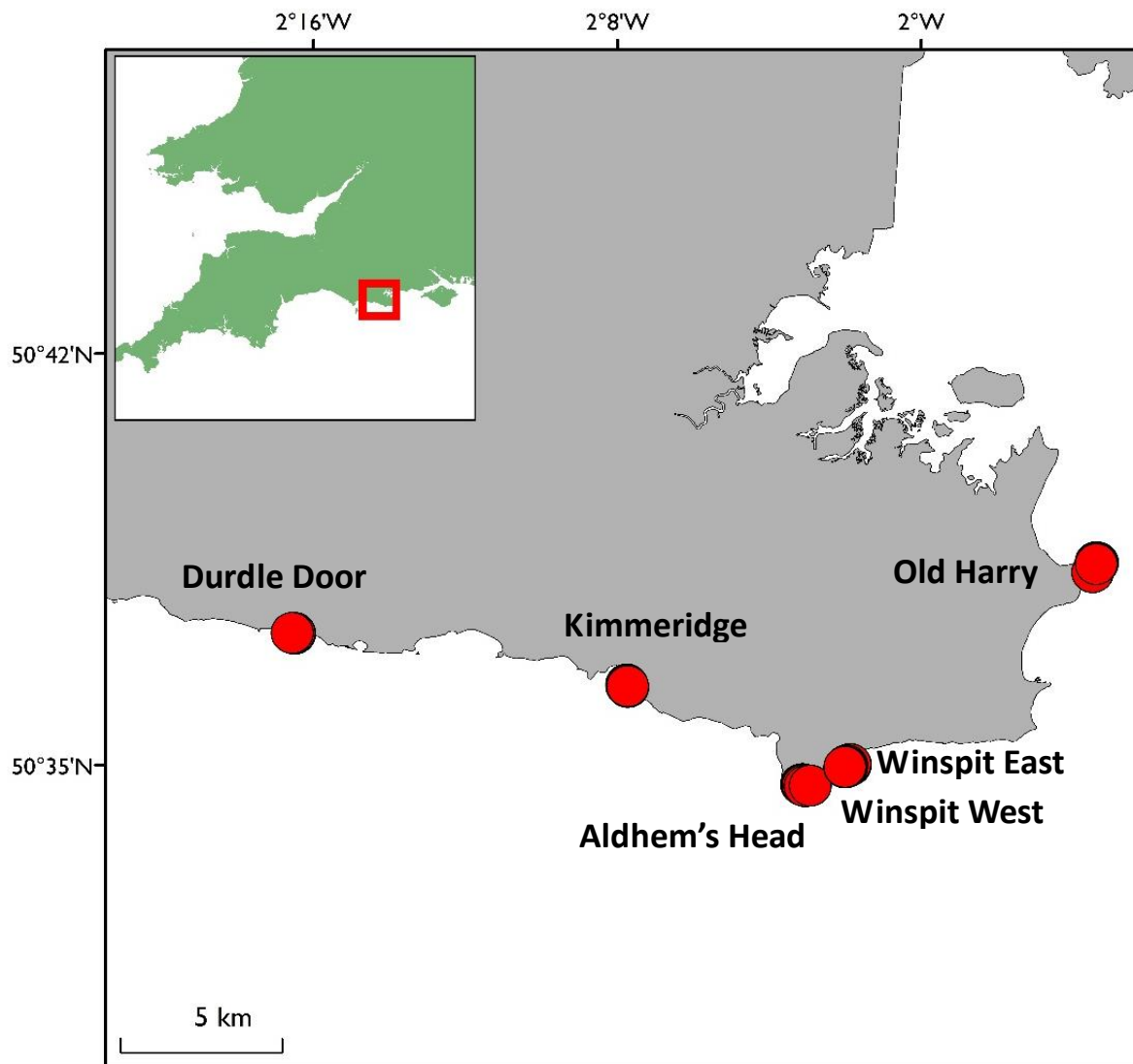


Figure 3.2. Map showing the location of natural *Brassica oleracea* plants from six natural populations in Dorset. Ten glucosinolates from indole and aliphatic glucosinolate groups were identified for 20 plants from each population.

*Extraction and Analysis of Intact Glucosinolates.* After harvest, plant material was immediately frozen in liquid nitrogen, lyophilized to dryness, and ground to a powder using tungsten beads with a diameter of 2 mm and a vibrating-ball micro mill (based on the Retsch MM300 ball mill). Tissue disruption was carried out for 4 min at a vibration frequency of 25 s<sup>-1</sup>. A 10mg aliquot of plant leaf tissue was transferred into 1.5 ml microcentrifuge tubes. Thermal degradation of glucosinolates has been observed at temperatures over 50°C (Mohn et al. 2007), therefore extraction took place in cold, 80% methanol (Goodey et al. 2015): 400 µl of extraction medium (80% methanol containing internal standards umbelliferone, 7.2 µg/ml, and linamarin 1.25 µg/ml) was added to plant samples. Lyophilized tissue samples were incubated on ice for 30 minutes with vortex mixing every ten minutes, followed by 15 minutes of sonication. After centrifugation (10 min at 16,100 x g, 4 °C), the supernatant was decanted and filtered through a 0.45 µm (PVDF) syringe filter (Chromacol, Welwyn Garden City, UK).

To generate a list of glucosinolates compounds present in the *B. oleracea* populations (Table 3.2), metabolite profiling was performed using a QToF 6520 mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. Glucosinolates fragment to produce sulfate product ions that can be used as identifiers for glucosinolates (Barbieri et al. 2008; Fabre et al. 2007; Mellon et al. 2002; Rochfort et al. 2008; Tian et al. 2005). Sample extract (5 µl) was loaded onto a Zorbax StableBond C18 1.8 µm, 2.1 x 100 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The following gradient was used: 0 min – 0% B; 3 min – 0% B; 7 min – 20% B; 20 min – 100% B; 30 min – 100% B; 31 min – 0% B. The flow rate was 0.25 ml min<sup>-1</sup> and the column temperature was held at 35 °C for the duration.



The source conditions for electrospray ionization were as follows: gas temperature was 325 °C with a drying gas flow rate of 9 liter min<sup>-1</sup> and a nebuliser pressure of 35 psig. The capillary voltage was 3.5 kV. The fragmentor voltage was 115 V and skimmer 70 V. Scanning was performed using the auto MS/MS function. Survey scan rate was 4 scans s<sup>-1</sup> and MS/MS scan rate was at 3 scans sec<sup>-1</sup> with a sloped collision energy of 3.5 V/100 Da with an offset of 5 V.

Quantitative glucosinolate analysis was performed using multiple reaction monitoring (MRM) on an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, USA). The HPLC system was the same as that used for QToF analyses. Sample extracts (15 µl) were loaded onto a Zorbax Eclipse Plus C18 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA) with fragmentor voltage and collision energies optimized for each compound (Table 4.2). The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 25 min – 100% B; 27 min – 0% B. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 liter min<sup>-1</sup>, nebuliser pressure 35 psig, capillary voltage ±4 kV.

Glucosinolates were identified by accurate mass and the presence of characteristic product ions (Table 3.2) (Barbieri et al. 2008; Fabre et al. 2007; Mellon et al. 2002; Velasco et al. 2008). The presence of sinigrin and progoitrin were further confirmed by comparing retention times and ion fragmentation patterns with pure standards (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany and Merck KGaA, Darmstadt, Germany). In the absence of standards for all the glucosinolates, concentrations are expressed as peak areas normalized by peak area of the internal standard, linamarin.

*Statistical Analyses.* Statistical analyses were performed in R 3.0.2 (R Core Team 2013). All analyses were performed using log-transformed glucosinolate concentrations: this transformation normalized the residuals and satisfied the homogeneity of variance assumption for standard parametric statistical tests. In order to assess host utilization in relation to plant glucosinolate profile, we asked whether plants with and without identified herbivore species in each wild population differed in chemotype syndrome, using linear discriminant analysis with herbivore presence as a binary classifier in the R package, MASS (Venables & Ripley 2002). We interpreted the dominant linear discriminant function in each population following scaling of all log-transformed glucosinolate concentrations to zero mean and unit standard deviation.

Generalized linear mixed models were used to determine how herbivore host utilization was related to discriminant function, and to a number of physical plant characteristics and ecological variables. Plant morphological characteristics included as fixed effects were plant rosette size (log-transformed and scaled to have 0 mean and unit standard deviation), leaf colour and toughness. Population was included in models as a random effect. Models containing a complete set of candidate variables were constructed in R 3.0.2 (R Core Team 2013) using lme4 (Bates et al. 2014). Models were fitted with binomial error structures to reflect the binary data on infestation by each herbivore species.

Model selection was conducted using an information-criterion approach, using the R package MuMIn (Bartoń 2013) to rank models based on AICc (correction for small sample sizes). We considered the best supported models to be within  $\Delta 7$  AICc units of the top model (see appendix for model tables). These

models were then model-averaged using the ‘model.av’ function in MuMin. The confidence intervals from top model parameters were used to plot model average figures.

## RESULTS

*Herbivore species.* Herbivores identified in each population were the crucifer specialist butterflies, *Pieris brassicae* and *P. rapae* (present as eggs oviposited on plants) and the specialist aphid *Brevicoryne brassicae*. Generalist species included the snail *Cornu aspersum*, and the aphid *Myzus persicae*. However, *M. persicae* was detected very rarely and was not included in subsequent analyses due to poor statistical power.

*Glucosinolates.* Ten glucosinolates from two different glucosinolate groups, aliphatic and indole, were identified (see Table 3.2).

Table 3.2. Transitions and data acquisition parameters used in HPLC-MSMS for quantification of detected glucosinolates.

Glucosinolate group	Common name	Chemical name	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Fragmentor voltage (V)	Collision energy (V)	Retention time (min)
Aliphatic	Gluconapoleiferin	4-Hydroxy-4-Pentenyl glucosinolate	402	97	100	19.1	8.2
Aliphatic	Progoitrin	(2R)-2-Hydroxy-3-butenyl glucosinolate	388	97	100	18.6	2.8
Aliphatic	Gluconapin	3-Butenyl glucosinolate	372	97	100	18.2	7.3
Aliphatic	Sinigrin	2-Propenyl glucosinolate	358	97	100	15	1.6
Aliphatic	Glucoiberberin	3-methylthiopropylglucosinolate	406	97	100	19.2	12.9
Aliphatic	Glucobrassicinapin	4-Pentenylglucosinolate	386	97	100	18.5	14.9
Aliphatic	Glucocapparin	Methylglucosinolate	332	97	100	16.6	3.2
Aliphatic	Glucocheirolin	3-Methylsulfonylpropyl	438	97	100	20.3	8.8
Indolic	Glucobrassicin	3-Indolymethyl glucosinolate	447	97	100	20.7	1.79
Indolic	Hydroxyglucobrassicin	4-hydroxyindol-3-ylmethylglucosinolate	463	97	100	11.3	9.7

*Herbivore species and glucosinolate profiles.* Linear discriminant analysis of 120 plants from across six populations in Dorset significantly separated plants with and without *P. rapae* (Fisher's exact test, odds ratio = 9.95, n=120, P= <0.001), snails (Fisher's exact test, odds ratio = 5.192, n=120, P= 0.010) and *B. brassicae* (Fisher's exact test, odds ratio = 4.487, n=120, P= 0.042) but was only near-significant for *P. brassicae* (Fisher's exact test, odds ratio = 1.94, n=120, P= 0.098). In each analysis, a single discriminant function (LD1) that best separated plants with and without each herbivore species was provided. However, the contribution of each identified glucosinolate to LD1 varied with herbivore species (figure 3.2). For *P. rapae*, plants with higher levels of glucocheirolin and glucobrassicin, but low levels of gluconapin and glucocapparin, contribute the most to separation between host and non-host plants. *P. brassicae* also showed a preference for plants with high concentrations of glucocheirolin, but with the addition of progoitrin and gluconapin and, unlike *P. rapae*, low concentrations of glucobrassicin were favoured. *B. brassicae* plant separation was best explained by high levels of progoitrin and low concentrations of glucobrassicinapin, glucocheirolin and glucobrassicin. For the generalist snail, LD1 was predominantly driven by a combination of high glucocheirolin and glucobrassicin, with lower contributions from gluconapin and glucocapparin.

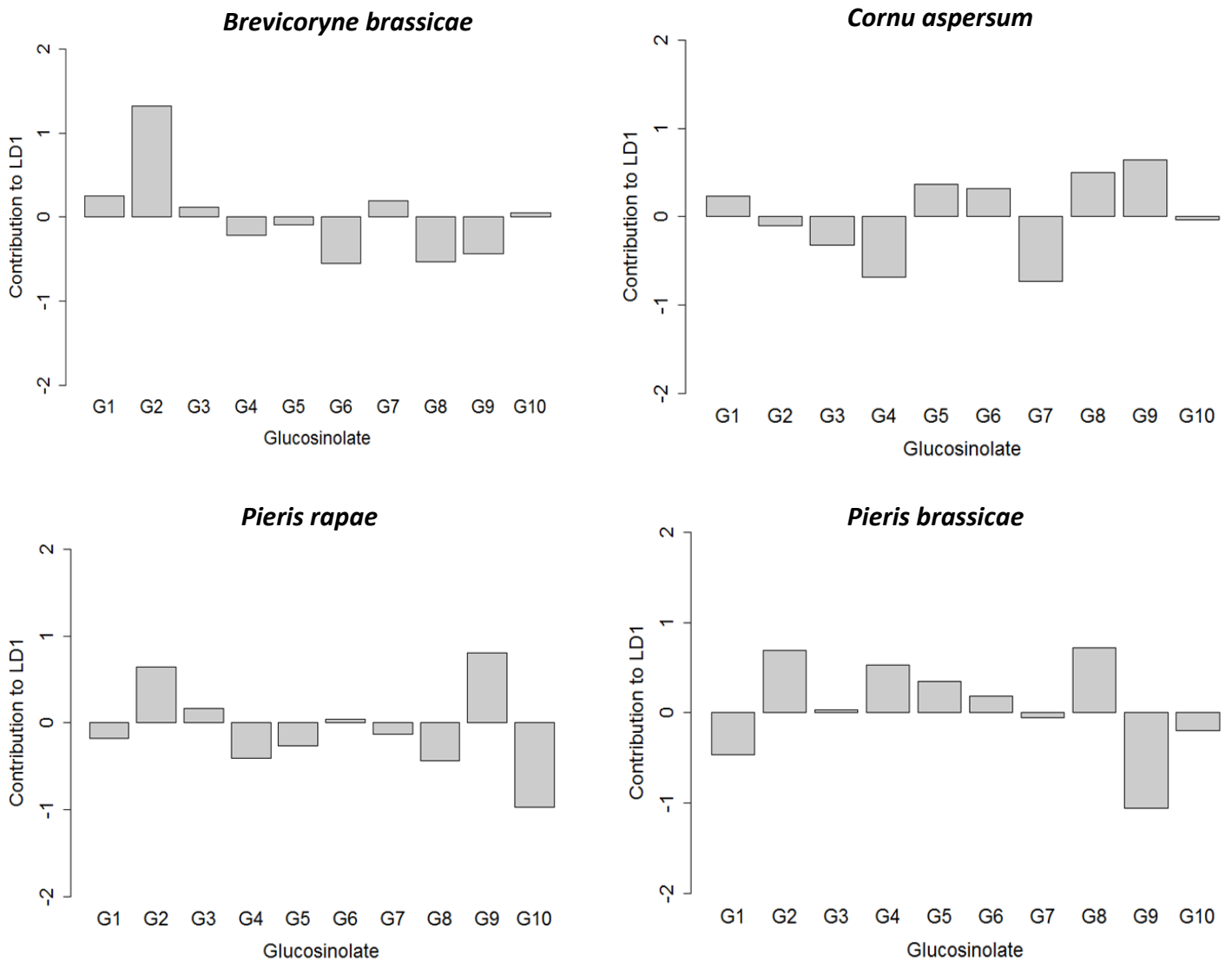


Figure 3.2. A linear discriminant function of glucosinolate profiles classifying 120 plants across six wild populations in Dorset on the presence or absence of *Pieris rapae* and *P. brassicae* eggs, common snails, *Cornu aspersum* and the cabbage aphid, *Brevicoryne brassicae*. For each species, the contribution of each identified glucosinolate to LD1 varies. GS1= sinigrin, GS2= progoitrin, GS3= glucoiberberin, GS4= gluconapin, GS5= gluconapoleiferin, GS6= glucobrassicinapin, GS7= glucocapparin, GS8= glucocheirolin, GS9= glucobrassicin, GS10=hydroxyglucobrassicin.

*Factors influencing host plant utilization by herbivores.* The probability of infestation by an herbivore species was modelled with its corresponding discriminant function and plant morphological characteristics. The probability of infestation for each herbivore species increased with its corresponding LD1 (figure 3.3). *C. aspersum*, *P. brassicae* and *B. brassicae* all showed a positive response to increasing rosette size, whereas *P. rapae* preferred to oviposit on smaller plants (see appendix 1 for AIC herbivore preference models).

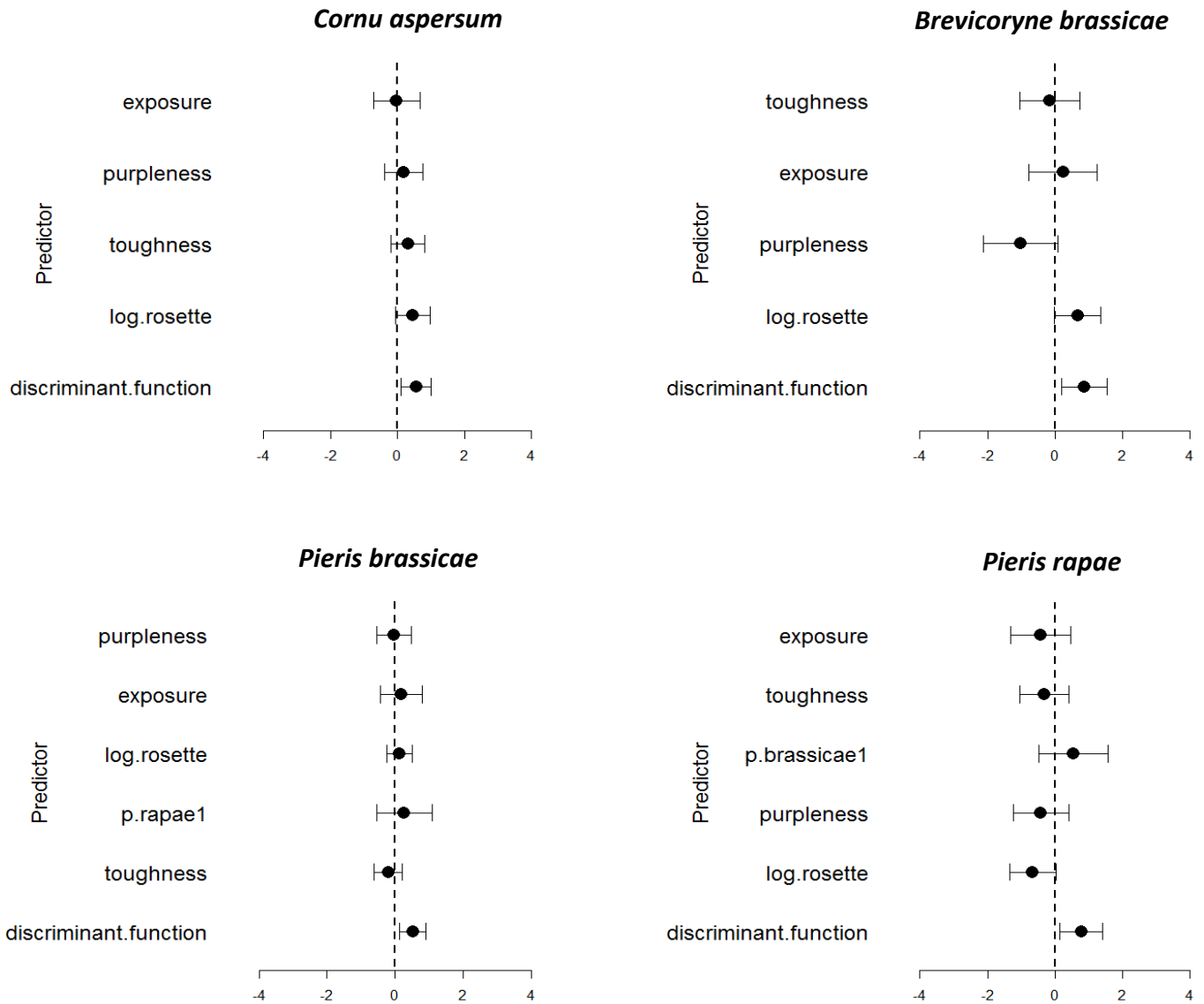


Figure.3.3. Confidence interval plots showing model averaged estimates for best supported models, within  $\Delta 7$  AICc units of the top model. Predictors with confidence intervals that span zero (x axis) do not have good explanatory power. For all herbivore species, discriminant function is the best predictor of host use. Increasing rosette size also has good explanatory power for *Cornu aspersum* and *Brevicoryne brassicae*; however *Pieris rapae* prefers smaller plants.



## DISCUSSION

Few studies have evaluated the responses of herbivores to intraspecific variation in a suite of plant defence chemicals in the ecological setting in which they take place (Moyes and Raybould 2001; Bidart-Bouzat and Kliebenstein 2008; Newton et al. 2009b; Zangerl et al. 2015). In natural populations of *B. oleracea* we have demonstrated a relationship between the differential contribution of ten glucosinolates, representing two chemical groups, to plant phenotype and host utilization by specialist and generalist herbivores. For each species, discriminant functions were the best predictor of host choice, even for *P. brassicae* where LDA on chemotype data alone could not significantly separate plants with eggs from those without. Though not a significant predictor in isolation, it becomes important when other plant traits were included. Whilst correlational associations do not mean causation, our results are strongly suggestive of differential host choice mediated by variation in plant glucosinolate profiles.

We predicted that if there was fluctuating selection maintaining variation in plant glucosinolate profiles, we should find species-specific preferences for glucosinolate phenotypes. We found that the contribution of individual compounds to desirable plant profile varied for each herbivore species. For example, *P. brassicae* females preferred plants with low contributions of sinigrin and glucobrassicin to chemical phenotype, and high contributions from glucocheirolin and progoitrin. In laboratory assays, sinigrin and glucobrassicin have been previously documented as positive stimuli for both large and small white oviposition, as has glucocheirolin, though to a lesser degree (van Loon et al. 1992; Huang and Renwick 1994; Stadler et al. 1995; Smallegange et al. 2007b).

Our results contradict those of Newton et al (2009) who reported a positive association between *P. brassicae* and plants producing sinigrin, although this effect was only observed at the intra-population scale and was not consistent through time.

*P. rapae* females also preferred plant phenotypes with high contributions of progoitrin, but differed from *P. brassicae* in their responses to indole glucosinolates, preferring positive contributions of glucobrassicin and low hydroxyglucobrassicin. *P. rapae* produce a nitrile specifier protein that redirects glucosinolate hydrolysis from highly toxic isothiocyanates to less harmful nitriles that are then excreted by feeding larva (Wittstock et al. 2004). However, experiments have shown that the larvae of *P. rapae* develop poorly on plants producing high concentrations of neoglucobrassicin, a derivative of glucobrassicin (Gols et al. 2008). Therefore, there may be limitations to a specialist's ability to cope with increasing quantities of particular compounds, and low concentrations of these chemicals may indicate plant quality to females, enabling them to make optimal host choices for their progeny (Jaenike 1978).

For the specialist brassica aphid, *B. brassicae*, host plant choice appeared to be driven by negative contributions of the aliphatic compounds glucobrassicinapin and glucocheirolin, and the indole glucosinolate glucobrassicin. Similarly to butterfly responses, aphids also preferred plant phenotypes with high contributions of progoitrin. These results both agree, and conflict, with previous findings. For example, other studies have found negative, or no association between glucobrassicin concentration and infestation by *B. brassicae* (Moyes et al. 2000; Mewis et al. 2006; Goodey et al. 2015); glucobrassicinapin has been shown to be positively correlated with intrinsic rate of increase (Cole 1997b). Differences in outcomes between studies may be a reflection of the variation in specific aphid

response to glucosinolates measured across studies, from intrinsic rate of increase, to simple counts of abundance or presence/absence on host plants. Our finding of an attraction of *B. brassicae* to progoitrin is also in direct contradiction of the pattern we found in a previous study, where we showed a negative relationship between progoitrin concentration and the probability of infestation (Goodey et al. 2015). One reason for this difference might be due to the fact that the plant tissues sampled varied between these studies: here we measured glucosinolate concentrations of leaves, as opposed to concentrations within flowering stems measured for our previous study. Glucosinolate concentrations have been shown to vary significantly between different tissues within individual plants, and foraging herbivores have been shown to be able to detect and respond to this variation (Shelton 2005; Smallegange et al. 2007a). In addition, these studies were undertaken in different populations and at different spatial scales.

Generalist herbivores are typically shown to have negative responses to high glucosinolate concentrations (for review, see Winder and Wittstock 2011). We therefore expected snail infestation to decrease with increasing concentrations of all identified glucosinolates. Instead, we found preference for a suite of glucosinolates, including positive contributions from glucocheirolin and glucobrassicin, and negative contributions from gluconapin and glucocapparin. However, Newton et al (2009) reported a positive response of snails to sinigrin presence that was consistent at different spatial scales, and across seasons and years. The disparity between our results may reflect the fact that we analysed differences in herbivore responses to quantitative, rather than qualitative variation, across a range of glucosinolates. Thus the preference for sinigrin previously reported may actually be driven by aversion to other compounds.

There are several reasons why species-specific responses to glucosinolate may exist. For example, whilst brassica specialists have evolved adaptations to circumvent potentially deleterious glucosinolate breakdown products (Winde and Wittstock 2011), variation in the mechanisms used by each species may result in different susceptibilities to various glucosinolates (Newton et al. 2009b). Alternatively, if host plant chemistry affects the location efficiencies of predators and/or parasites (De Moraes et al. 1998), insect herbivores may be using plant chemicals to avoid natural enemies (Jeffries and Lawton 1984). However, the glucosinolate profiles analysed here were measured during the process of herbivory, therefore some of the observed variation in species preferences may describe plant responses to infestation, rather than herbivore preference for plant chemotype (Hilker and Meiners 2006).

We predicted that if there was opposing selection for glucosinolate profile, we should be able to group specialists and generalists according to similar responses. Although snails did differ from specialists, unfortunately this was the only generalist species recorded at densities high enough to enable analysis with reasonable statistical power. However, we did find overlapping preferences for specialist herbivores: the aliphatic glucosinolate progoitrin was a positive correlate of infestation across all specialist herbivore species. While this was observed in all specialists, preferences across a wide range of glucosinolates showed variation between each species, regardless of dietary range, suggesting that selection may be highly variable. It is therefore plausible that the significant inter-population variation in glucosinolate concentration and composition observed in wild brassica species could be maintained by fluctuating selection, but that there may be directional selection on progoitrin concentration by brassica specialists. Our results recommend a focus on experimental studies of progoitrin as the key driver of specialist herbivory in wild Brassica populations, and on the survey of a broader suite of generalist

herbivores to better determine whether generalists share preference for, or aversion to, certain glucosinolates, or certain plant chemotypes.

In this study we included plant traits other than glucosinolates previously suggested to be important for host plant selection. No herbivore exhibited a significant response to exposure or leaf toughness. However this may be a reflection of our method of measurement, which was very subjective, and leaf toughness has been shown to deter herbivory for other plant-herbivore systems (Coley 2013). Our measurement of leaf colour was similarly flawed, as our assessment of leaf “purpleness” was based on human colour perception and is therefore not necessarily a biologically relevant category (Chittka and Doring 2007). All herbivores surveyed did show a preference for larger rosette size, with the exception of *P. rapae* which preferred to lay eggs on smaller plants. We postulate here that females may be responding to some unmeasured physiological plant trait associated with smaller plant size, or may be avoiding competition for limited resources; unlike *P. brassicae* females, which lay large batches of eggs whose feeding larvae may require more than one plant in order to complete development, small white butterflies lay eggs singly (Chew 1980). Thus a preference for smaller plants may enable *P. rapae* to avoid competition for limited resources.

We now know that herbivore species have clear, species-specific preferences for chemical profiles of wild cabbage plants in natural populations. Future research should aim to place these results in the context of fluctuating selection pressures. Following Newton et al (2009, 2010), the goal should be to determine whether species’ preferences are consistent or fluctuate through time and space, and whether

species' relative abundances change accordingly. These are key building blocks for the theory that Brassica metabolite diversity is maintained by spatial or temporal fluctuations in the type and intensity of herbivory.

## **CHAPTER FOUR**

**BUTTERFLY PREFERENCE FOR CABBAGE CHEMOTYPES VARIES ACROSS POPULATIONS**

**BUT DOES NOT PREDICT LARVAL PERFORMANCE**

## ABSTRACT

Plant secondary metabolites provide the basis for host plant selection in many herbivorous insects. Despite numerous studies demonstrating the importance of these plant chemicals for selection among host plant species, and subsequent differential offspring performance, there is a lack of knowledge of how intraspecific variation in secondary metabolite profile affects links between preference and performance. Here we used a classic system in plant-insect interactions, the large white butterfly *Pieris brassicae* and its host plant *Brassica oleracea*. We compared the glucosinolate profiles of wild plants with and without eggs across five natural cabbage populations in Dorset. In a common garden experiment, we reared sister-broods of large white larvae collected from the surveyed plants, placing them on either plants descended from their population of origin, or from a different population. We found evidence for chemotype preferences for egg-laying females both within and across populations, but no relationship was found between these preferences and the survival or pupal weight of their offspring. Instead, we found a significant effect of natal plant ancestry: larval survival was greater when reared on plants descended from their own population. Our results show that glucosinolates appear to be important cues for egg-laying females at different spatial scales, but that other heritable plant traits are more important for developing larvae.



## INTRODUCTION

Plant secondary metabolites play a key role in host plant selection by phytophagous insects (Dethier 1954; Fraenkel 1959). The host range of many insect herbivores is limited, and oligophagous species in particular are often restricted to plants containing taxonomically distinct classes of secondary compounds (Jaenike 1990; Zangerl et al. 1993). The behavioural decision to accept or reject a potential host for oviposition or feeding is therefore largely based on plant chemical profile (Jaenike 1990). Accurate assessment of host plant suitability is essential, especially for ovipositing females whose offspring have limited mobility and are dependent on the accurate choice of their mother (Jaenike 1978). Thus, as female choice carries significant consequences for her offspring (Bossart 2003), it is expected that egg laying females should seek to maximize their fitness by using variation in plant chemistry to discriminate between host and non-host species, and poor and high quality plants (Jaenike 1978; Thompson 1991; Mayhew 1997). A positive relationship between female preference and offspring performance is therefore predicted by what is commonly referred to as the preference-performance hypothesis (PPH) (Jaenike 1978).

Results reported from a range of insect taxa suggest females generally exhibit a preference for plants which support, at a minimum, adequate offspring performance (Mayhew 1997; Gripenberg et al. 2010). However, the strength of this relationship is often weak, and in some cases the link is absent altogether (Gripenberg et al. 2010). Of the many tests of the PPH, most are conducted between plant species within an insect's host range (but see Horner and Abrahamson 1992; Craig et al. 1999; Ryan and Bidart-Bouzat 2014), assuming that each plant represents an ecologically equivalent host species. However, many insect populations are restricted geographically or behaviorally to a single plant species (Fox and

Morrow 1981). So, for egg-laying females in nature, the degree to which intraspecific variation in plant chemistry drives differential host choice is an ecologically relevant question. Given the widespread within species variation in secondary metabolite profiles (Kroymann 2011), a species-level approach may mask significant effects of individual plant variation on the preference-performance relationship.

In this study, we utilize the large white butterfly, *Pieris brassicae*, in order to investigate the PPH in relation to intraspecific variation in host plant secondary metabolites. Like other white butterflies, *P. brassicae* is largely restricted to feeding on cruciferous plants containing characteristic glucosinolate metabolites (Chew 1980). The role of these sulfur- and nitrogen-containing compounds in mediating *Pieris*-plant interactions is well documented: glucobrassicin and sinigrin have been shown to be important for host recognition, egg-laying and larval feeding stimulation and larval growth rate (van Loon et al. 1992; Huang and Renwick 1994; Stadler et al. 1995; Renwick and Lopez 1999; Smallegange et al. 2007b). However, previous research has predominantly studied the stimulatory effects of single compounds, whereas a balance of positive and negative signals perceived by an insect in nature is likely to be important in assessing host quality (Dethier 1982; Huang and Renwick 1994). For example, a previous study investigating the performance of *P. brassicae* in response to glucosinolate variation found that neoglucobrassicin, an indole compound, had a negative effect on larval development (Harvey et al. 2011), suggesting not all glucosinolates are beneficial for offspring.

In addition, the majority of previous studies have been conducted between brassica species or cultivars, or use non-host substrates treated with extracted glucosinolates (Renwick and Radke 1988; Du et al. 1995; Stadler et al. 1995). In the wild, female butterflies will be confronted with variation both in the presence and/or absence of specific compounds, and differences in relative concentrations of a suite of

compounds from different structural groups. Types and amount of aliphatic glucosinolates, for example, are under strong genetic control and are highly heritable (Kliebenstein et al. 2001b), resulting in variable, yet relatively predictable phenotypes among populations. However, indole glucosinolates are highly plastic and may be rapidly induced in response to abiotic and biotic stressors in a plant's local environment (Agrawal et al. 2002). Hence the plant chemotype to which the ovipositing mother is exposed is complex, and determined by both genetic and environmental effects. If plant chemotype affects larval fitness, then the egg and time-limited mother is likely to use plant glucosinolates in order to select the best quality host plants for her offspring.

In natural populations of the wild cabbage *Brassica oleracea*, significant intraspecific variation in glucosinolate profile has been shown across a variety of spatial scales (Mithen et al. 1995b; Moyes et al. 2000; Newton et al. 2009b). We surveyed plants, from five spatially separated populations, for the presence and absence of *P. brassicae* eggs in order to reveal a preferred glucosinolate phenotype. We deliberately chose a paired “case-control” design to maximize the signal of chemotype preference by standardizing plants according to a suite of other phenotypic traits including leaf toughness, colour and exposure. Given previous findings (Renwick and Radke 1983; Huang and Renwick 1994), we predicted that sinigrin and glucobrassicin would contribute significantly to the preferred plant chemotype, but that high concentrations of other detected indole glucosinolates would be avoided. We hypothesized that these preferences would be adaptive, and therefore tested the prediction that offspring would perform better on plants expressing the glucosinolate profile chosen by their mothers. We speculate that the spatial arrangement and isolation of host plant populations will influence local selection pressures, and therefore may yield links between preference and performance at a population, rather than geographic level.

## MATERIALS AND METHODS

*Female Preference Assay.* Oviposition preferences were assessed in June 2013 across five wild populations of *B. oleracea* in Dorset, England (figure 4.1). In order to isolate the effects of plant chemistry on host plant selection, ten pairs of plants were selected in each population. The two members of each pair differed, to the best of our ecological knowledge, only in the presence or absence of *P. brassicae* eggs: after a plant with eggs was located, a neighbouring egg-free plant was selected, no more than two meters away and matched for location, size (as measured by rosette and basal stem diameter), leaf colour and exposure. We chose this matched-pair design to help deal with the non-chemical features of plant phenotype and environment that could disguise signal of chemical preferences in ovipositing mothers. Leaf tissue samples (1cm<sup>2</sup> sections excised from five randomly selected leaves) were harvested from both egg and non-egg plants, and immediately flash frozen in liquid nitrogen. Egg batches were removed (n= ten batches for each population) from their host plants and transplanted to one of two treatments in a common garden population in order to assay larval performance.

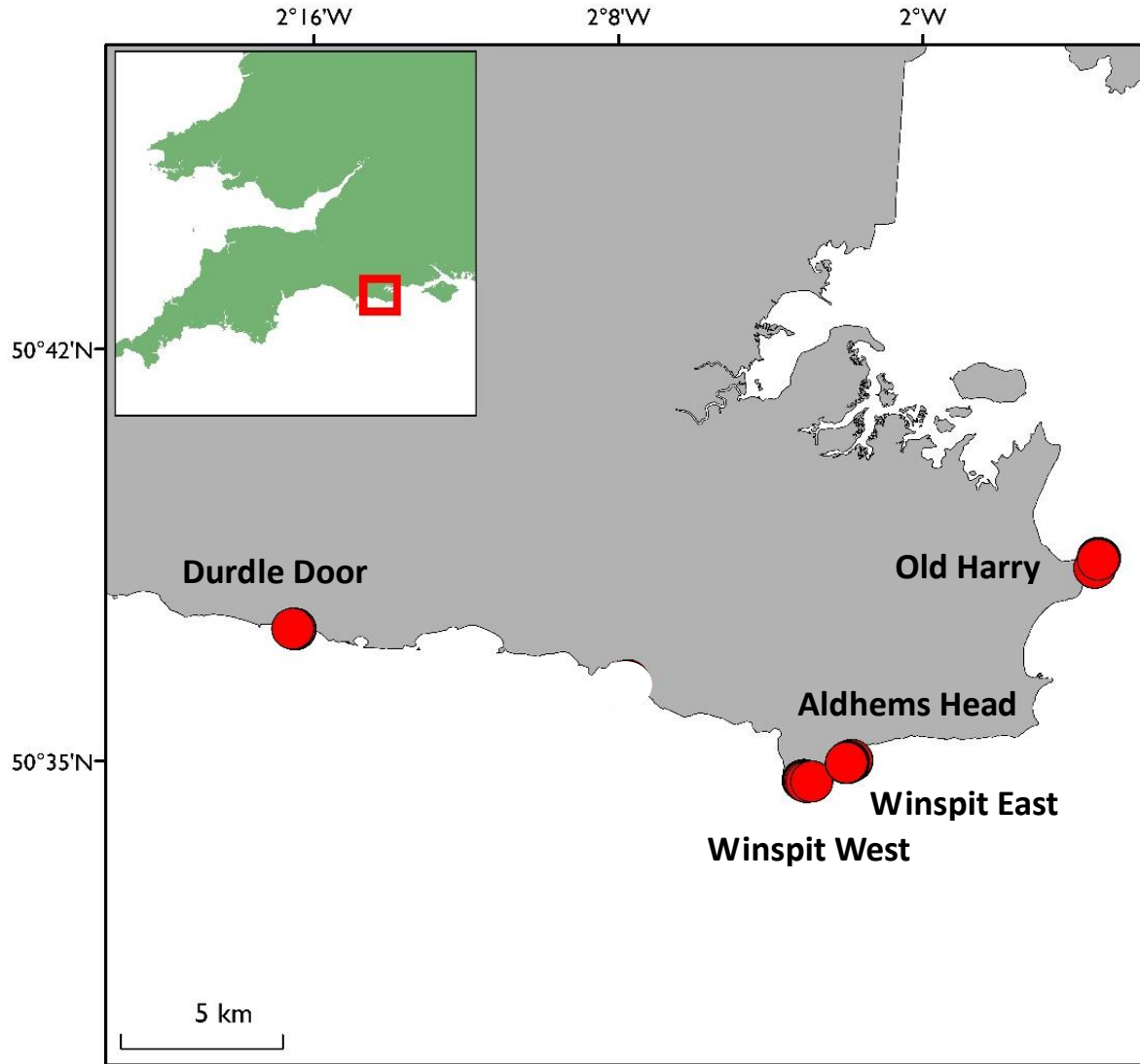


Figure.4.1. Map showing the location of natural *Brassica oleracea* plants in Dorset. Glucosinolates were identified for 20 plants from each population, ten with *Pieris brassicae* egg clutches, and ten without, in order to investigate female oviposition preference.

*Larval Performance.* Progeny performance was assayed on *B. oleracea* using a split-brood design. Assays were conducted in a common garden using plants reared from seeds collected from the same wild populations five years previously (figure 4.1). Harvested egg batches from wild populations in Dorset were halved, and approximately ten eggs allocated to one of two plant ancestry treatments: ‘home’ plants originating from the progeny’s natal population, or ‘away’ plants, originating from a randomly selected ‘foreign’ population in the South West. In order to set aside any effects of natural enemies on performance, egg batches were bagged in organza, and once the larvae had hatched were moved onto fresh foliage on their allocated host plant as required. Larvae were checked daily for mortality and accumulated exuviae was removed from the bags. Larvae were reared until they reached the pupal stage, at which point they were collected and weighed to give pupal mass. Larval survival was calculated as the number of larvae that lived to pupation.

*Extraction and Analysis of Intact Glucosinolates.* After harvest, plant material was immediately frozen in liquid nitrogen, lyophilized to dryness, and ground to a powder using tungsten beads with a diameter of 2 mm and a vibrating-ball micro mill (based on the Retsch MM300 ball mill). Tissue disruption was carried out for 4 min at a vibration frequency of  $25\text{ s}^{-1}$ . A 10mg aliquot of plant leaf tissue was transferred into 1.5 ml microcentrifuge tubes. Thermal degradation of glucosinolates has been observed at temperatures over  $50^{\circ}\text{C}$  (Mohn et al. 2007), therefore extraction took place in cold, 80% methanol (Goodey et al. 2015): 400  $\mu\text{l}$  of extraction medium (80% methanol containing internal standards umbelliferone,  $7.2\text{ }\mu\text{g/ml}$ , and linamarin  $1.25\text{ }\mu\text{g/ml}$ ) was added to plant samples. Lyophilized tissue samples were incubated on ice for 30 minutes with vortex mixing every ten minutes, followed by 15 minutes of sonication. After centrifugation (10 min at  $16,100\text{ x g}$ ,  $4^{\circ}\text{C}$ ), the supernatant was decanted and filtered through a  $0.45\text{ }\mu\text{m}$  (PVDF) syringe filter (Chromacol, Welwyn Garden City, UK).

To generate a list of glucosinolates compounds present in the *B. oleracea* populations (Table 4.1), metabolite profiling was performed using a QToF 6520 mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. Glucosinolates fragment to produce sulfate product ions that can be used as identifiers for glucosinolates (Barbieri et al. 2008; Fabre et al. 2007; Mellon et al. 2002; Rochfort et al. 2008; Tian et al. 2005). Sample extract (5  $\mu$ l) was loaded onto a Zorbax StableBond C18 1.8  $\mu$ m, 2.1 x 100 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The following gradient was used: 0 min – 0% B; 3 min – 0% B; 7 min – 20% B; 20 min – 100% B; 30 min – 100% B; 31 min – 0% B. The flow rate was 0.25 ml min<sup>-1</sup> and the column temperature was held at 35 °C for the duration. The source conditions for electrospray ionization were as follows: gas temperature was 325 °C with a drying gas flow rate of 9 liter min<sup>-1</sup> and a nebuliser pressure of 35 psig. The capillary voltage was 3.5 kV. The fragmentor voltage was 115 V and skimmer 70 V. Scanning was performed using the auto MS/MS function. Survey scan rate was 4 scans s<sup>-1</sup> and MS/MS scan rate was at 3 scans sec<sup>-1</sup> with a sloped collision energy of 3.5 V/100 Da with an offset of 5 V.

Quantitative glucosinolate analysis was performed using multiple reaction monitoring (MRM) on an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, USA). The HPLC system was the same as that used for QToF analyses. Sample extracts (15  $\mu$ l) were loaded onto a Zorbax Eclipse Plus C18 3.5  $\mu$ m, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA) with fragmentor voltage and collision energies optimized for each compound (Table 4.1). The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100%

B; 25 min – 100% B; 27 min – 0% B. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 liter min<sup>-1</sup>, nebuliser pressure 35 psig, capillary voltage ±4 kV.

Glucosinolates were identified by accurate mass and the presence of characteristic product ions (Table 4.1) (Barbieri et al. 2008; Fabre et al. 2007; Mellon et al. 2002; Velasco et al. 2008). The presence of sinigrin and progoitrin were further confirmed by comparing retention times and ion fragmentation patterns with pure standards (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany and Merck KGaA, Darmstadt, Germany). In the absence of standards for all the glucosinolates, concentrations are expressed as peak areas normalized by peak area of the internal standard (linamarin), quantity of extraction medium, and tissue weight.



Table 4.1. Transitions and data acquisition parameters used in HPLC-MSMS for quantification of detected glucosinolates.

Glucosinolate group	Common name	Chemical name	Precursor ion ( <i>m/z</i> )	Product ion ( <i>m/z</i> )	Fragmentor voltage (V)	Collision energy (V)	Retention time
Aliphatic	Gluconapoleiferin	4-Hydroxy-4-Pentenyl glucosinolate	402	97	100	19.1	8.2
Aliphatic	Progoitrin	(2R)-2-Hydroxy-3-butenyl glucosinolate	388	97	100	18.6	2.8
Aliphatic	Gluconapin	3-Butenyl glucosinolate	372	97	100	18.2	7.3
Aliphatic	Sinigrin	2-Propenyl glucosinolate	358	97	100	15	1.6
Aliphatic	Glucoiberberin	3-methylthiopropylglucosinolate	406	97	100	19.2	12.9
Aliphatic	Glucobrassicinapin	4-Pentenylglucosinolate	386	97	100	18.5	14.9
Aliphatic	Glucocapparin	Methylglucosinolate	332	97	100	16.6	3.2
Aliphatic	Glucocheirolin	3-Methylsulfonylpropyl	438	97	100	20.3	8.8
Indolic	Glucobrassicin	3-Indolymethyl glucosinolate	447	97	100	20.7	1.79
Indolic	Hydroxyglucobrassicin	4-hydroxyindol-3-ylmethylglucosinolate	463	97	100	11.3	9.7

*Statistical Analyses.* Statistical analyses were performed in R 3.0.2 (R Core Team 2013). All analyses were performed using log-transformed glucosinolate concentrations: this transformation normalized the residuals and satisfied the homogeneity of variance assumption for standard parametric statistical tests.

In order to assess female preference in relation to host plant chemistry, we asked whether plants with and without *P. brassicae* eggs in each wild population differed in chemotype syndrome, using linear discriminant analysis with egg presence as a binary classifier using the R package MASS (Venables & Ripley 2002). We interpreted the dominant linear discriminant function in each population, and across all populations, following scaling of all log-transformed concentrations to zero mean and unit standard deviation.

The resulting discriminant functions for each population provided single summary covariates that sort glucosinolate profiles according to maternal preference. In order to assess larval performance in relation to this preference, we projected the chemotypes of the selected common garden plants onto the discriminant functions determined by our preference analysis. This sorted the “rearing” plants according to maternal preference. We predict that if the PPH holds true, larvae should survive and grow better on preferred than on non-preferred chemotypes. We then conducted generalized linear mixed models in lme4 (Bates et al. 2014), with larval survival or pupal weight as dependent variables, and “preference” discriminant scores and plant ancestry as fixed effects. Larval brood and natal populations were included as random effects. Models were fitted with binomial or Gaussian error structures respectively, to test the influence of discriminant functions on larval survival and pupal weight.

## RESULTS

*Female preference.* Linear discriminant analysis (LDA) of plants from across all sampled populations in Dorset correctly predicted the egg category of plants in 68% of cases, providing a single discriminant function (LD1) that best separated plants with eggs from plants without (Fisher's exact test,  $n=100$ ,  $P=0.027$ ). LD1 increased with increasing concentrations of progoitrin and glucocheirolin, with decreasing sinigrin, glucoiberberin and glucobrassicin, and was little influenced by the remaining compounds (figure 4.2). Hence across populations, female large whites oviposited on plants producing high concentrations of the aliphatic compounds progoitrin and glucocheirolin, but were less likely to lay on plants producing high concentrations of sinigrin, glucoiberberin and glucobrassicin.

LDA was also successful at classifying plants when conducted at a population level: LD1 correctly predicted the egg category of plants in Winspit East (85% classification accuracy,  $P = 0.003$ ), Aldhems Head, Durdle Door, Old Harry (all 85% accuracy,  $P = 0.005$ ), with the exception of plants in Winspit West for which Fisher's exact test was nonsignificant (25% accuracy,  $P = 0.07$ , all populations  $n = 20$ ). The contribution of each compound to LD1 varied between populations (figure 4.3). Therefore whilst females preferred to oviposit on one glucosinolate phenotype over another, this preference varied with all populations except for Winspit West (figure 4.3).

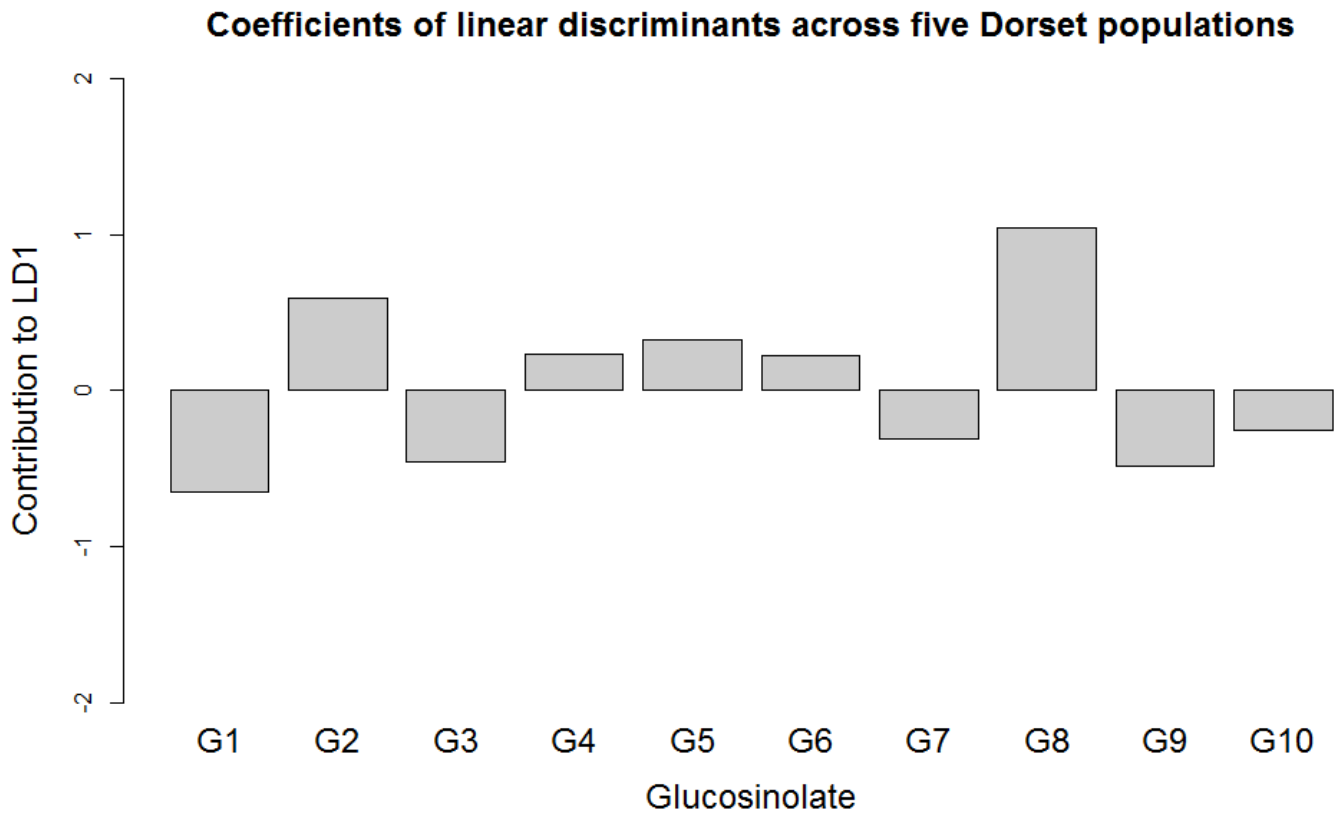


Figure.4.2. Linear discriminant function of glucosinolate profiles from plants with and without *P. brassicae* eggs across five wild populations in Dorset. There is a positive loading towards progoitrin (GS2) and glucocheirolin (GS8) and negative loading towards sinigrin (GS1), glucoiberberin (GS3) and glucobrassicin (GS9). The remaining glucosinolates gluconapin (GS4), gluconapoleiferin (GS5), glucobrassicinapin (GS6), glucocapparin (GS7) and hydroxyglucobrassicin (GS10) contribute negligibly to the discriminant function.

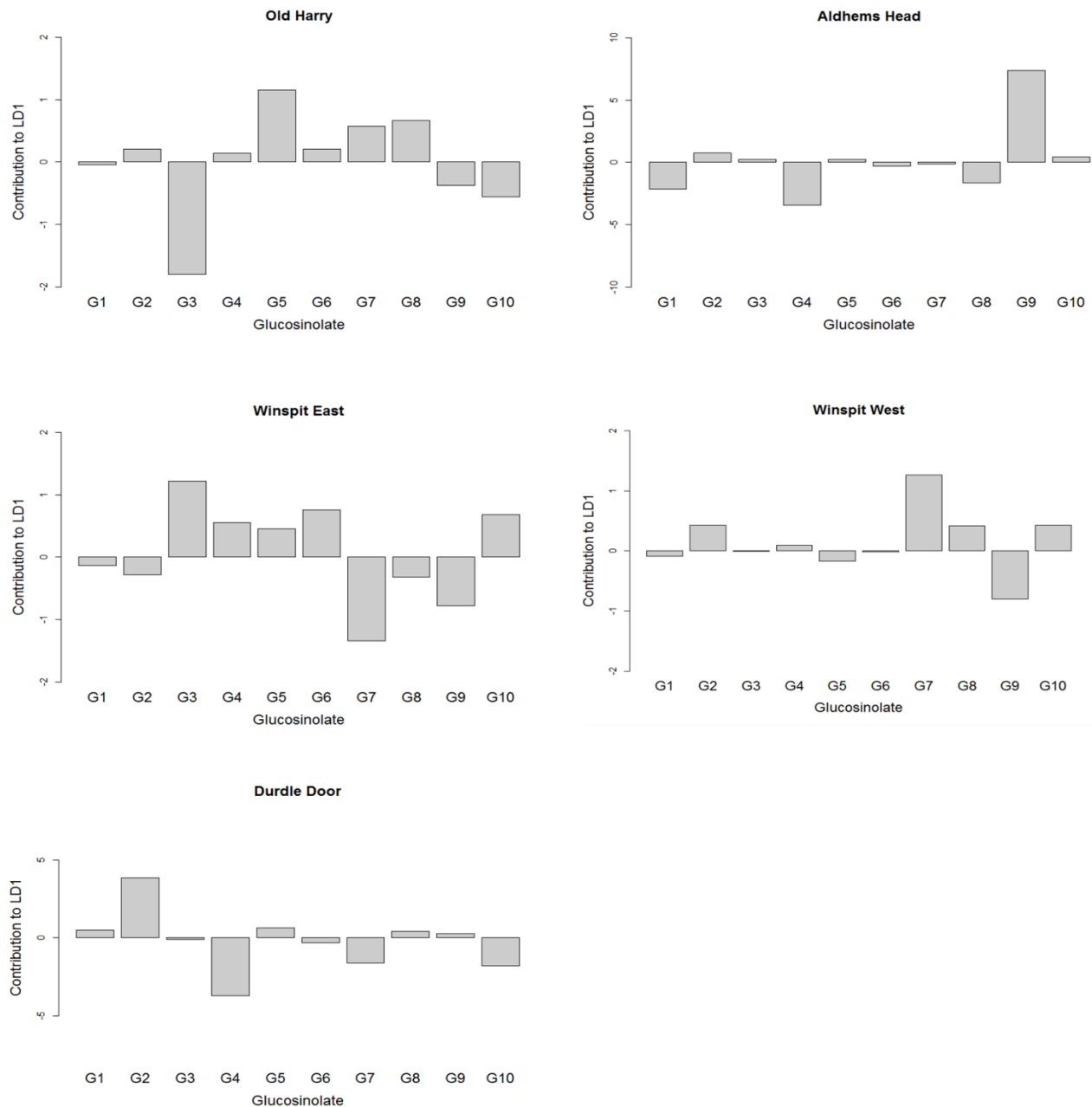


Figure.4.3. Linear discriminant function of glucosinolate profiles from plants with and without *P. brassicae* eggs within five wild populations in Dorset. Preferred glucosinolate phenotype varies in each population, but general trends emerge: progoitrin (GS2) contribution to LD1 is normally positive, glucoiberverin (GS3) contributes little to preference for plants in Aldhem’s Head or Durdle Door, but contributes highly for remaining populations, though the direction changes from positive to negative. In all populations apart from Aldhem’s Head, glucocheirolin (GS7) contributes highly, but again the direction of the contribution changes. GS1= sinigrin, GS2= progoitrin, GS3= glucoiberverin, GS4= gluconapin, GS5= gluconapoleiferin, GS6= glucobrassicinapin, GS7= glucocapparin, GS8= glucocheirolin, GS9= glucobrassicin, GS10= hydroxyglucobrassicin.

*Larval performance.* Pupal weight was not effected by plant ancestry ( $x_1^2 = 0.53$ ,  $P = 0.62$ ), or glucosinolate discriminant function (within population:  $x_1^2 = 0.79$ ,  $P = 0.25$ ; across populations:  $x_1^2 = 0.55$ ,  $P = 0.61$ ). Nor was there an effect of discriminant function on survival (within populations  $x_1^2 = 0.61$ ,  $P = 0.60$ ; across populations:  $x_1^2 = 0.23$ ,  $P = 0.63$ ). However, there was a significant, positive effect of plant ancestry on larval survival ( $x_1^2 = 0.03$ ,  $P = 0.02$ , figure 4.4).

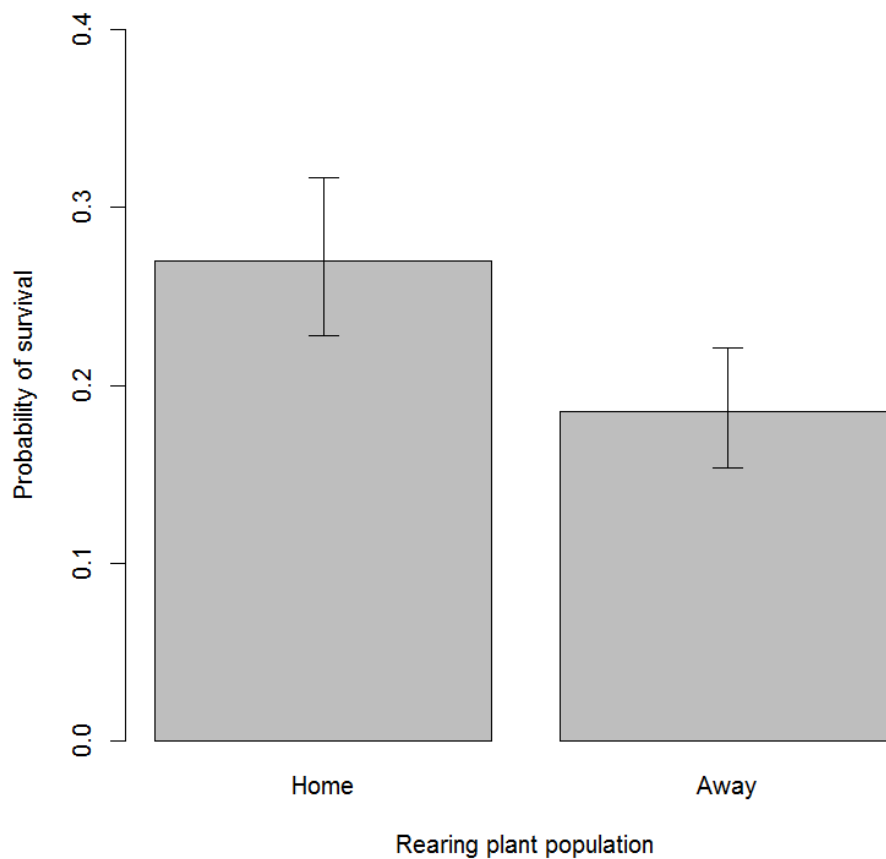


Figure.4.4. Plant ancestry had a significant effect on the probability of *Pieris brassicae* larvae surviving to pupation in a common garden experiment: larval survival was greater on plants descended from a brood's home population, than on plants descended from other *Brassica oleracea* populations in the UK.

## DISCUSSION

Plant secondary metabolites, such as glucosinolates, have long been of interest due to their major role in plant-insect interactions, particularly in guiding host plant acceptance or rejection (Jaenike 1990).

Female host choice carries significant consequences for her offspring (Bossart 2003), therefore a tight link is expected between female preference and offspring performance (Jaenike 1978). However, there are a lack of studies investigating responses to intraspecific variation in plant profiles across different spatial scales, and even fewer which study within species plant preferences and its consequences for larval performance (Horner and Abrahamson 1992; Craig et al. 1999; Cronin et al. 2001). In this study, we found female large white exhibited differential preferences for glucosinolates at the inter- and intra-population scale. However, in contrast to the predictions of the preference-performance hypothesis, we found no discernible link between preferred chemotype and offspring performance. Instead, we present evidence that for larvae reared in a common garden, survival was strongly affected by the ancestry of their host plant.

A number of studies have reported the role of glucosinolates, in particular sinigrin and glucobrassicin, as positive stimuli for white butterflies (Huang and Renwick 1994; Du et al. 1995; Stadler et al. 1995). We therefore predicted that ovipositing *P. brassicae* females in the wild would prefer to lay their eggs on plants expressing high concentrations of these compounds in their chemotypes. We also predicted, given previous reports of negative effects of some indole compounds on larval performance (Harvey et al. 2007; Gols et al. 2008), that preferences may reflect a balance of positive and negative stimuli. However, our results showed that across populations, females preferred to lay eggs on plants with low concentrations of sinigrin and high concentrations of progoitrin and glucocheirolin.

The differences between our results and those previously reported may reflect differences in the type of study: the majority of research on butterfly responses has been conducted in laboratory settings with extracted compounds, whereas our study investigates female preferences to intact plants in natural populations. Attraction to glucosinolate profiles across wild populations of *B. oleracea* has also been found for another crucifer specialist herbivore, the seed weevil *Ceutorhynchus assimilis* (Moyes and Raybould 2001).

Analysis of female responses within plant populations however, revealed considerable variation in glucosinolate preferences. Concentrations of progoitrin and glucocheirolin were generally positive in each population, though their contribution to preferred profiles varied. In no populations did sinigrin contribute significantly to preferred plant profile. Conversely, in a similar study Newton et al (2009) reported a positive association between *P. brassicae* and plants producing sinigrin within populations. However, their study measured qualitative glucosinolate variation, not quantitative differences. In each population, a balance of positive and negative glucosinolate concentrations was observed, but there was no consistency in which compounds contributed to this pattern. A previous study investigating female *P. napi* oleracea responses to aliphatic and aromatic glucosinolates also found that, whilst females responded strongly to compounds in these groups, their relative concentrations to plant profile showed no clear pattern (Huang and Renwick 1994).

In order to investigate the potential for glucosinolates to structure herbivore communities; which in turn would provide evidence for selection by herbivores as a mechanism maintaining variation in plant defences both within and between populations, surveys need be carried out at different spatial scales (Moyes and Raybould 2001; Newton et al. 2009b). We found female responses to glucosinolate



phenotype did vary with scale. There are several ecological reasons why female attraction to glucosinolate profiles may exhibit such variation. First, there are likely to be differences in the distances at which individual glucosinolates can be perceived. For example, it has been suggested that the sinigrin and its breakdown products are highly volatile (Mitchell 1977), which suggests that this compound may be detectable at a distance. Therefore, females searching for host plants across populations may be attracted to an area by longer-range allelochemicals. Once a suitable patch of plants has been located, glucosinolate variation detectable at the leaf surface may guide females in making local choices.

Second, larval or adult experience may result in individual differences in glucosinolate preferences (Davis 2007). Host plant use in some populations of the butterfly *Battus philenor* has been shown to reflect individual variation in foraging behaviour due to adult experience (Rausher and Rapaj 1983; Courtney et al. 2013). Additionally, when larvae of the crucifer specialist *Phyllotreta xylostella* were reared on *Arabidopsis thaliana* genotypes with variable glucosinolate profiles, emergent females exhibited increased oviposition preferences for the same genotypes (Ryan and Bidart-Bouzat 2014). This suggests preference may not be completely innate, but involves some learning, which may enable mothers to match their preferences to local variation in offspring performance (Cunningham and West 2008; Ryan and Bidart-Bouzat 2014).

Such a scenario is possible if local preferences indicate local adaptation to glucosinolate profiles. There is potential for genotype-by-environment interactions, a pre-requisite for local adaptation (Kawecki and Ebert 2004), in these populations. Considerable variation has been documented for *B. oleracea* plants, even between populations in close proximity (Mithen et al. 1995b; Moyes et al. 2000; Harvey et al. 2007; Newton et al. 2009b). This variation has been shown to remain in common garden populations,

suggesting that local conditions generate highly divergent, heritable chemotypes (Harvey et al. 2007). Given the stability of these populations, which have persisted for decades, potentially centuries (Wichmann et al. 2009), and the long-lived nature of these plants, there is the potential for herbivores to become adapted to a particular suite of predictable, local glucosinolate phenotypes.

If there is a relationship between glucosinolate profile and larval performance, then there is a strong selective advantage for females to oviposit on plants with the same profile (Wolfson 1980). Other studies have shown that plant population can have significant consequences for *P. brassicae* larval development. For example, Harvey et al (2011) found that pupal mass and development time varied significantly among Dorset populations of *B. oleracea*. Furthermore, poor development correlated with increasing concentrations of the indole glucosinolate, neoglucobrassicin. Yet another study reported no effects of glucosinolate variation on the performance of specialist herbivores (Poelman et al. 2008a). In order to isolate the effects of glucosinolate phenotype on *P. brassicae* larvae, we reared broods in a common garden setting. However we found no relationship between glucosinolate profiles preferred by females, either within or between populations, and offspring performance.

There are several reasons we may have failed to detect a relationship. First, it could be that the importance of plant glucosinolate profiles depends on the life stage of the offspring. For example, early instars of *P. rapae* were shown to be negatively affected by increasing concentrations of host glucosinolates when reared on *B. napus*, but this effect disappeared for later instars (Rotem et al. 2003). In this study we did not measure growth rate, development time or emergent adult size and the effect of glucosinolates may be restricted to one of these stages. Additionally, whilst female host choice initially determines the food plant of her offspring, larvae of *P. brassicae* become increasingly mobile

throughout their development (Lemasurier 1994). Our finding of a lack of correspondence may indicate that natural selection favours oviposition on plants best for egg and early instar development, and that a mother's choice is mediated by the subsequent host selection of her offspring. We therefore recommend future studies measure larval performance across all life stages.

Finally, we note evidence that Brassicas adjust their expression of secondary metabolites in response to oviposition by specialist butterflies (van Loon et al. 1992). We therefore cannot deny the possibility that differences in chemotype between plants with and without eggs describe plant responses rather than maternal herbivore preferences. In fact, adaptive responses by plants could negate and even reverse the predictions of the preference-performance hypothesis (Dicke 2000; Hilker and Meiners 2006). Future studies should carefully disentangle “preference” from “response to being selected” to determine whether chemical differences between attacked and non-attacked plants are caused by plant or herbivore responses.

Whilst we found no effect of glucosinolate preferences on larval performance, we did find that offspring survival was higher when reared on common garden plants descended from a brood's population of origin. This suggests that *P. brassicae* larval performance is linked to an unknown heritable plant trait that varies at a population level. Studies of other insect herbivore species have shown differential effects of plant traits other than secondary chemistry on performance, such as foliar protein content (Scheirs et al. 2003), leaf toughness (Larsson and Tenow 1987) and leaf thickness (Picoaga et al. 2003).

Furthermore, in a study exploring the performances of specialist crucifer insects on different brassica cultivars, it was found that whilst performance varied depending on cultivar, this variation was not

related to glucosinolate profiles (Poelman et al. 2008a). The authors therefore concluded that other, unidentified secondary chemicals or plant traits may be responsible.

In summary, the data presented here indicate that female *Pieris brassicae*, a specialist butterfly of glucosinolate containing plants, exhibits preferences for variable plant glucosinolate profiles at different spatial scales, but that this preference does not correspond with the performance of their offspring. Further work should analyse glucosinolate profiles of preferred plants before and after egg deposition, and explore parameters of larval performance across developmental instars. We also provide evidence that indicates the existence of an unknown plant trait that may be mediating this relationship: larval broods survived better on 'home' plants, suggesting other plant traits may be important selective agents for this species at a local scale. The extent of butterfly movement between these long-lived plant populations is not known, therefore further work should investigate patterns of butterfly population genetics (Via 1991) to explore the potential for local adaption to plant chemotypes, or other aspects of plant phenotype.

## CHAPTER FIVE

### APHIDS PICK THEIR POISON: SELECTIVE SEQUESTRATION OF PLANT CHEMICALS AFFECTS HOST PLANT USE IN A SPECIALIST HERBIVORE

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## ABSTRACT

In some plant-insect interactions, specialist herbivores exploit the chemical defences of their food plant to their own advantage. *Brassica* plants produce glucosinolates that are broken down into defensive toxins when tissue is damaged, but the specialist aphid, *Brevicoryne brassicae*, uses these chemicals against its own natural enemies by becoming a “walking mustard-oil bomb”. Analysis of glucosinolate concentrations in plant tissue and associated aphid colonies reveals that not only do aphids sequester glucosinolates, but they do so selectively. Aphids specifically accumulate sinigrin to high concentrations while preferentially excreting a structurally similar glucosinolate, progoitrin. Surveys of aphid infestation in wild populations of *Brassica oleracea* show that this pattern of sequestration and excretion maps onto host plant use. The probability of aphid infestation decreases with increasing concentrations of progoitrin in plants. *B. brassicae* therefore appear to select among food plants according to plant secondary metabolite profiles, and selectively store only some compounds to use against their own enemies. Our results demonstrate chemical and behavioural mechanisms that help to explain evidence of geographic patterns and evolutionary dynamics in *Brassica*-aphid interactions.

**Key Words**-*Brassica oleracea*, *Brevicoryne brassicae*, excretion, glucosinolate, host-plant selection, sequestration.

## INTRODUCTION

Plants and insect herbivores are engaged in a biochemical arms race involving reciprocal evolution of defense and counter defense (Ehrlich and Raven 1964; Fraenkel 1959). An increasing body of evidence reveals that some specialists exploit plant secondary metabolites, sequestering them as defenses against their own natural enemies (Nishida 2002; Opitz and Müller 2009). If certain plant toxins provide a fitness advantage to the herbivore, natural selection should favor plant genotypes that either decrease toxin production (Malcolm and Zalucki 1996), or produce new defenses. In this case, the sequestration behavior of the herbivore will not reflect the defense chemotype of the host plant. Instead, specialist herbivores will sequester some plant defensive compounds that they are adapted to co-opt, but will excrete or avoid defenses that they are unable to handle. Furthermore, assuming that sequestration and excretion are costly, herbivores should be selected to prefer food-plants that produce the co-opted defense and avoid plants producing compounds they tend to excrete.

Like other important plants in the order Brassicales, including *Arabidopsis thaliana*, wild cabbage *Brassica oleracea* is defended by the glucosinolate-myrosinase system. This system consists of structurally diverse compounds, classified according to their amino acid precursors into aliphatic, indolic or aromatic glucosinolates, which are stored separately from hydrolyzing enzymes known as myrosinases (Fahey et al. 2001; Halkier and Gershenzon 2006). Glucosinolates and myrosinase are brought together when tissue damage, such as that caused by herbivore feeding, breaks down compartmentalization (Halkier and Gershenzon 2006). Depending on the substrate structure, hydrolysis then converts the glucosinolate into various biologically active products including highly reactive isothiocyanates (ITCs) (Halkier and Gershenzon 2006; Winde and Wittstock 2011). To negate the

harmful effects of these compounds, several generalist herbivores detoxify ITCs (Pentzold et al. 2014; Winder and Wittstock 2011; Wittstock et al. 2004) whereas the majority of specialist species investigated so far appear to process their diet in ways that prevent ITC formation (Wittstock et al. 2003).

Due to its non-chewing mode of feeding, the Brassica specialist aphid *B. brassicae* is able to sequester intact glucosinolates from its host plant, accumulating them to very high concentrations in the hemolymph (Bridges et al. 2002; Kazana et al. 2007). Subsequent hydrolysis of these sequestered compounds into ITCs is enabled through the action of a coevolved aphid myrosinase, stored separately in thorax and head muscles (Bridges et al. 2002; Kazana et al. 2007; Pontoppidan et al. 2003). The breakdown products released when the aphid's body is damaged have been implicated in natural enemy defense and alarm signaling, leading to the conclusion that *B. brassicae* mimics and exploits the defense system of its host plant (Bridges et al. 2002; Kos et al. 2011).

A naïve interpretation of this exploitation of host defenses is that aphids simply mimic the chemical defense phenotype of their food-plants. However, recent evidence suggests that *B. brassicae* has selective sequestration patterns. For example, comparisons of total glucosinolate concentrations in plant tissue and aphid carcasses have revealed that sequestration of compounds may be selective at the level of glucosinolate group, with aphids accumulating high levels of constitutive aliphatic glucosinolates, but not of highly volatile, inducible indolic compounds (Bridges et al. 2002; Kazana et al. 2007; Kos et al. 2012). Recent evidence also suggests that the ability of aphids to metabolize glucosinolates varies among compounds. Aphid myrosinase exhibits differential activity towards glucosinolate substrates, with reduced enzymatic activity towards progoitrin compared to sinigrin (Francis et al. 2002).



Furthermore, aliphatic glucosinolates vary in their thermal stability (Macleod & Rossiter, 1986). The risk of chemical degradation into toxic by-products might make some glucosinolates unsuitable for sequestration and storage. The biochemical interaction between plant and aphid is therefore complex and may be influenced by small differences in glucosinolate chemical structure.

There is empirical evidence that aphids exert directional selection on the heritable chemotype of their host plants. Aliphatic glucosinolate structure is under genetic control, with the production of compounds with three carbon (3C) or four carbon (4C) side-chains regulated by alleles at the *GS-ELONG* locus (Kliebenstein et al. 2001a; Figure 5.1). Geographical patterns in *A. thaliana* defence chemotypes match closely with geographical patterns in the prevalence of *B. brassicae*. For example, in regions where *B. brassicae* is the prevalent aphid, populations are dominated by plants expressing 4C aliphatic glucosinolates (including progoitrin) (Züst et al. 2012). In a multi-generation selection experiment in the same study, it was found that infestation by *B. brassicae* caused plants expressing 4C chemotypes to outcompete plants expressing 3C glucosinolates (including sinigrin). These results suggest that 4C compounds have a detrimental effect on aphid performance (Züst et al. 2012). While this aphid species is able to exploit some of the glucosinolates from its host, it appears not to be adapted to all components of the plant defence chemotype. It is therefore expected that *B. brassicae* should tailor sequestration accordingly, selectively accumulating those glucosinolates which provide an adaptive function and excreting harmful compounds as waste.

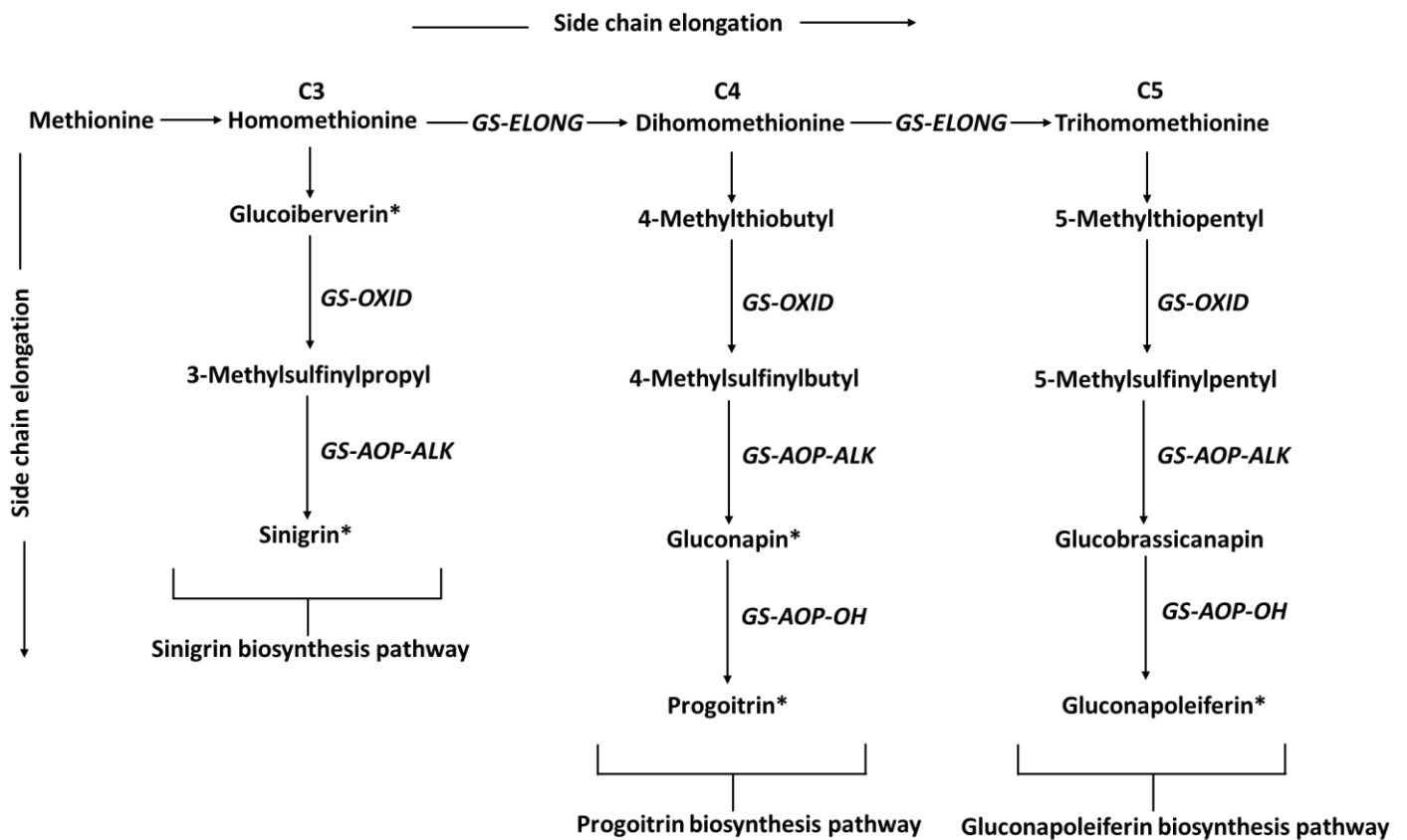


Figure 5.1. Biochemical pathways, genetic loci and alleles that yield structural diversity of aliphatic glucosinolates in *Brassica oleracea* (adapted from (Halkier and Gershenzon 2006; Kliebenstein et al. 2001a; Mithen 2001; Züst et al. 2012)). Common names in bold; chemical names in normal font or bracketed under common names; loci and alleles in italics. A methionine backbone yields an elongated carbon chain (transitions from C3 to C4 to C5 chain length mediated by alleles at locus *GS-ELONG*, Methylthioalkylmalate synthases (Kliebenstein et al. 2005) with side group modifications yielded by alleles at loci *GS-OXID* and *GS-AOP* (glucosinolate (S) oxygenase (Kliebenstein et al. 2001b) and 2-oxo acid- dependent dioxygenases (Halkier and Gershenzon 2006), respectively). In each pathway, allele *GS-AOP-ALK* yields the non-hydroxylated end product. Further modification of the C4 and C5 side chains is yielded by *GS-AOP-OH* to produce progoitrin and gluconapoleiferin. Asterisked glucosinolates are those assayed in this study.

Here we use targeted metabolite analysis to survey natural variation in aliphatic and indolic glucosinolate profiles of wingless *B. brassicae* aphids, of aphid honeydew and of the host plant, *B. oleracea*. We predicted that host plant sinigrin is accumulated to high concentrations within aphid bodies compared to host plant concentrations. We expected that 4C glucosinolates, such as progoitrin, are not accumulated to high concentrations and may even be actively excreted in aphid honeydew. We predicted that patterns of selective sequestration and excretion will map onto patterns of host plant infestation. We discuss our findings in the context of a coevolutionary arms race. All results come from wild cabbage populations, lending our study a high level of ecological relevance.

## MATERIALS AND METHODS

*Host Plant Selection and Tissue Harvesting.* In order to assess host plant selection, 250 plants were randomly selected from a wild population in Prussia Cove, Cornwall, UK (SW 55653 28034) during May 2013, and the presence or absence of *B. brassicae* colonies was recorded. Five leaves and a sample of inflorescence (flowering stem) were excised from each plant and flash frozen in liquid nitrogen for chemical analysis. To survey glucosinolate variation in aphids, 40 flowering plants with evidence of infestation were randomly selected from the Prussia Cove population in May 2014. These colonies were allowed to develop naturally for four weeks with exposure to natural enemies. As the distribution of plant glucosinolates is not restricted to phloem (Koroleva et al. 2000), and several species of aphid, including *B. brassicae*, have been shown to probe and ingest from other plant tissues such as xylem (Gabrys et al. 1997; Spiller et al. 1990), a sample was taken from the entire inflorescence that the colony was feeding on. Leaf tissue was harvested as previously mentioned. Honeydew was collected from aphid colonies by placing a cone of aluminium foil around the plant stem, immediately below the colony, for 24 hours. Aphid colonies were then bagged in organza, transferred to the laboratory and the number of adults counted. A subset of adults and nymphs (20 individuals of each lifestage where possible) were brushed into sample vials for extraction of glucosinolates. After harvest, all plant and insect material was immediately flash frozen in liquid nitrogen.

*Extraction and Analysis of Intact Glucosinolates.* Plant and aphid material was lyophilized to dryness, transferred into 1.5 ml microcentrifuge tubes and ground to a powder using tungsten beads with a diameter of 2 mm and a vibrating-ball micro mill (based on the Retsch MM300 ball mill). Tissue disruption was carried out for 4 min at a vibration frequency of 25 s<sup>-1</sup>.

It is typical to extract glucosinolates from plant tissues in hot or boiling methanol. However, thermal degradation of glucosinolates has been noted at extraction temperatures above 50 °C (Mohn et al. 2007). We therefore compared extraction in boiling 70% and cold (4 °C) 80% methanol and found no reduction in peak areas across all identified glucosinolates (appendix 2). The dried plant samples were weighed into 10 mg aliquots and 400 µl of extraction medium (80% methanol containing internal standards umbelliferone, 7.2 µg/ml, and linamarin 1.25 µg/ml) was added. Aphid carcasses, weighed to the nearest 0.01 mg, typically weighed much less than 10 mg (ranging from 0.1-0.5 mg dry weight). These lower-mass samples required smaller quantities of extraction medium (200 µl). As it was not possible to establish the mass of honeydew samples, the collection foil for each colony was washed in 50 µl of extraction medium. All plant, aphid and honeydew samples were incubated on ice for 30 min with vortex mixing every ten min, followed by 15 min of sonication. After centrifugation (10 min at 16,100 x g, 4 °C), the supernatants were decanted and filtered through 0.45 µm (PVDF) syringe filters (Chromacol, Welwyn Garden City, UK).

To generate a list of glucosinolates compounds present in the *B. oleracea* population (Table 5.1), metabolite profiling was performed using a QToF 6520 mass spectrometer (Agilent Technologies, Palo Alto, USA) coupled to a 1200 series Rapid Resolution HPLC system. Glucosinolates fragment to

produce sulfate product ions that can be used as identifiers for glucosinolates (Barbieri et al. 2008; Fabre et al. 2007; Mellon et al. 2002; Rochfort et al. 2008; Tian et al. 2005). Sample extract (5  $\mu$ l) was loaded onto a Zorbax StableBond C18 1.8  $\mu$ m, 2.1 x 100 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). Mobile phase A comprised 5% acetonitrile with 0.1% formic acid in water and mobile phase B was 95% acetonitrile with 0.1% formic acid in water. The following gradient was used: 0 min – 0% B; 3 min – 0% B; 7 min – 20% B; 20 min – 100% B; 30 min – 100% B; 31 min – 0% B. The flow rate was 0.25 ml min<sup>-1</sup> and the column temperature was held at 35 °C for the duration. The source conditions for electrospray ionization were as follows: gas temperature was 325 °C with a drying gas flow rate of 9 liter min<sup>-1</sup> and a nebuliser pressure of 35 psig. The capillary voltage was 3.5 kV. The fragmentor voltage was 115 V and skimmer 70 V. Scanning was performed using the auto MS/MS function. Survey scan rate was 4 scans s<sup>-1</sup> and MS/MS scan rate was at 3 scans sec<sup>-1</sup> with a sloped collision energy of 3.5 V/100 Da with an offset of 5 V.

Quantitative glucosinolate analysis was performed using multiple reaction monitoring (MRM) on an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, USA). The HPLC system was the same as that used for QToF analyses. Sample extracts (15  $\mu$ l) were loaded onto a Zorbax Eclipse Plus C18 3.5  $\mu$ m, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA) with fragmentor voltage and collision energies optimized for each compound (Table 5.2). The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 25 min – 100% B; 27 min – 0% B. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 liter min<sup>-1</sup>, nebuliser pressure 35 psig, capillary voltage  $\pm$ 4 kV.

Glucosinolates were identified by accurate mass and the presence of characteristic product ions (Table 5.1) (Barbieri et al. 2008; Fabre et al. 2007; Mellon et al. 2002; Velasco et al. 2008). The presence of sinigrin and progoitrin were further confirmed by comparing retention times and ion fragmentation patterns with pure standards (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany and Merck KGaA, Darmstadt, Germany). In the absence of standards for all the glucosinolates, concentrations are expressed as peak areas normalized by peak area of the internal standard (linamarin), quantity of extraction medium, and tissue weight. For the honeydew extracts we were unable to establish sample weight and therefore refer to honeydew peak areas normalized by the internal standard as glucosinolate “content”.

*Statistical Analyses.* Statistical analyses were performed in R 3.0.2 (R Core Team 2013). Analyses of plant and aphid carcass glucosinolates were performed using log<sub>10</sub>-transformed glucosinolate concentrations: this transformation normalized the residuals and satisfied the homogeneity of variance assumption for standard parametric statistical tests. We compared the concentrations of each glucosinolate among sample tissues using one-way analysis of variance followed by Tukey’s HSD test to compare between tissues without risking Type I errors. Comparisons were made between leaf, stem, nymphal aphid, and adult wingless aphid. The nymphal aphid samples provided a useful control that ensured any apparent sequestering behaviour of the adults was not simply an artefact of differences in extraction efficiency between plant and insect tissues during sample preparation.

To compare the content of glucosinolates found in honeydew to observed concentrations in aphid carcasses, we generated proportional compositional profiles by dividing the peak area of individual compounds by the total area across all glucosinolates, for each sample. Analysis of glucosinolates in aphid and honeydew samples was performed on arcsine-square-root transformed glucosinolate proportions. We compared the proportion of each glucosinolate among sample tissues using one-way analysis of variance and Tukey's HSD post-hoc comparisons.

We then asked whether naturally infested and uninfested plants ( $n = 226$  samples following the removal of 24 plants which did not express all identified glucosinolates) differed in chemotype, using linear discriminant analysis with aphid infestation as a binary classifier using the R package, MASS (Venables & Ripley 2002). We interpreted the dominant linear discriminant function following scaling of all log-transformed concentrations to zero mean and unit standard deviation. We then used a generalized linear model with binary error structure to test the influence of the discriminant function on the probability of infestation.



Table 5.1. Transitions and data acquisition parameters used in HPLC-MSMS for quantification of detected glucosinolates.

<b>Glucosinolate group</b>	<b>Common name</b>	<b>Chemical name</b>	<b>Precursor ion (<i>m/z</i>)</b>	<b>Product ion (<i>m/z</i>)</b>	<b>Fragment or voltage (V)</b>	<b>Collision energy (V)</b>	<b>Retention time (min)</b>
<b>Aliphatic</b>	Gluconapoleiferin	4-Hydroxy-4-Pentenyl glucosinolate	402	97	100	19.1	8.2
<b>Aliphatic</b>	Progoitrin	(2R)-2-Hydroxy-3-butenyl glucosinolate	388	97	100	18.6	2.8
<b>Aliphatic</b>	Gluconapin	3-Butenyl glucosinolate	372	97	100	18.2	7.3
<b>Aliphatic</b>	Sinigrin	2-Propenyl glucosinolate	358	97	100	15	1.6
<b>Indolic</b>	Glucobrassicin	3-Indolymethyl glucosinolate	447	97	100	20.7	1.79

## RESULTS

Across plant, aphid and honeydew samples, five glucosinolates were consistently present (Table 5.1). For each glucosinolate, concentrations varied significantly among tissues (one-way ANOVAs comparing relative concentrations among leaf, stem, nymphal aphid and adult wingless aphid: sinigrin,  $F_{3,169} = 50.15$ ; gluconapin,  $F_{3,169} = 10.98$ ; progoitrin  $F_{3,169} = 31.9$ ; gluconapoleiferin  $F_{3,169} = 46.84$ ; glucobrassicin  $F_{3,169} = 13.34$ ; all  $P < 0.001$ ). Tukey's HSD multiple comparisons of means showed significant differences between plant and aphid samples in glucosinolate concentration, with patterns that varied according to glucosinolate structure (Figure 5.2; tissues that differ significantly in content ( $P < 0.05$ ) differ in letter code).

Glucosinolate composition (measured as relative HPLC peak area of each glucosinolate) also varied significantly among nymphs, wingless aphids and excreted honeydew (sinigrin,  $F_{2,169} = 1157$ ; gluconapin,  $F_{2,169} = 7.31$ ; progoitrin  $F_{2,169} = 178.1$ ; glucobrassicin  $F_{2,169} = 12.12$ ; all  $P < 0.001$ ) with the exception of gluconapoleiferin ( $F_{2,169} = 0.524$ ,  $P = 0.594$ ). Tukey's HSD confirmed significant differences between wingless aphid and honeydew samples in relative concentrations of sinigrin and progoitrin ( $P < 0.01$ ) (Figure 5.3).

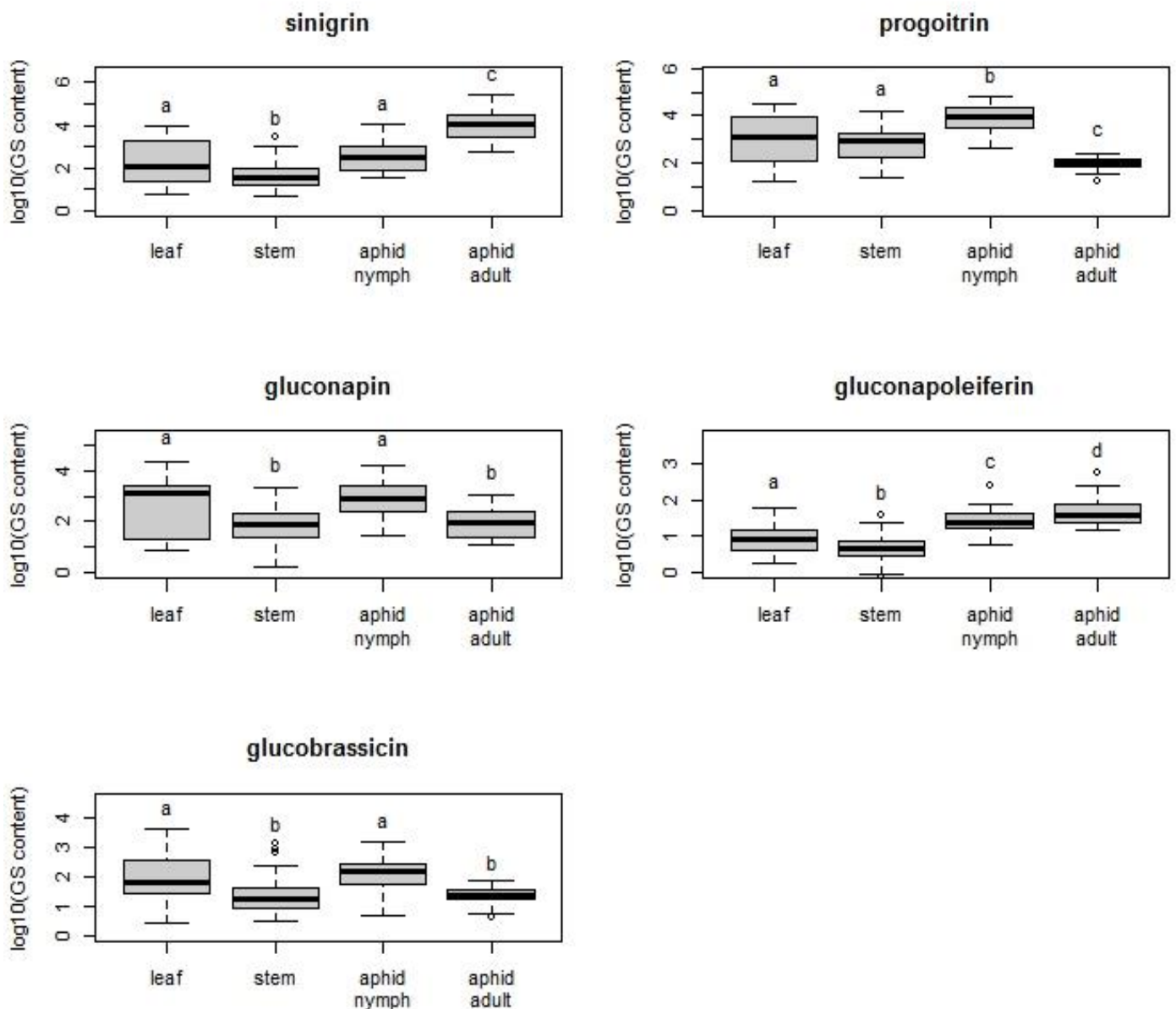


Figure 5.2. Log-transformed concentrations (measured as standardized peak area per  $\mu\text{l}$  extraction buffer per mg tissue) of aliphatic and indolic (glucobrassicin) glucosinolates in matched samples of plant leaf, flowering stem, aphid nymphs, and aphid wingless adults. Boxplots show range (whiskers), interquartile range (boxes), median (thick line), and putative outliers (open circles). Tissues that differ in letter code, per plot, differ significantly according to Tukey's HSD comparisons.

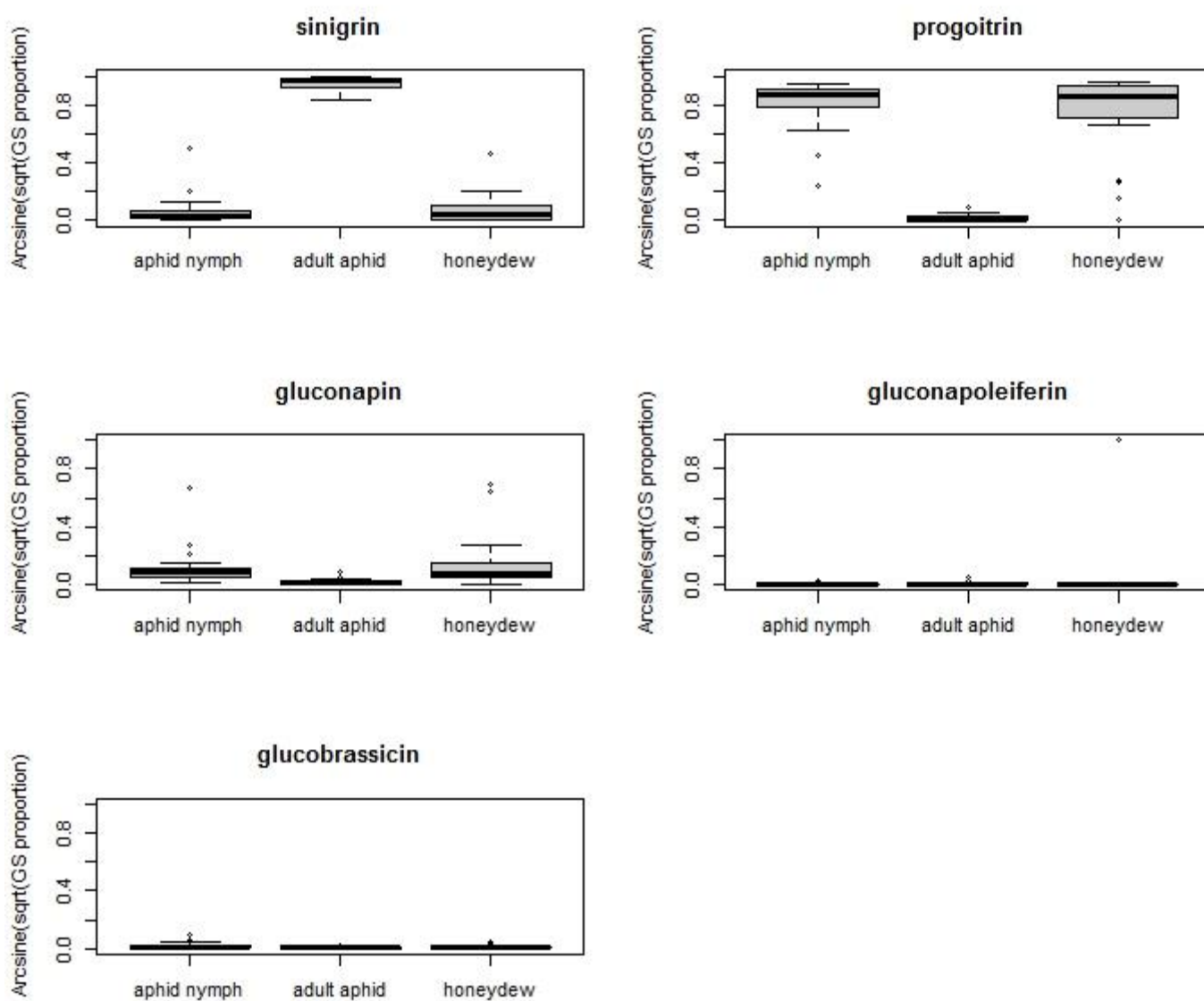


Figure 5.3. Arcsine–square-root- transformed proportions of aliphatic and indolic (glucobrassicin) glucosinolates in samples of aphid nymphs, aphid wingless adults and aphid honeydew. Boxplots show range (whiskers), interquartile range (boxes), median (thick line), and putative outliers (open circles). According to Tukey’s HSD comparisons, significant differences were found among tissues for sinigrin and progoitrin proportions in adult wingless aphids compared to honeydew.

Our key and most striking results were that adult aphids contained greatly increased concentrations of sinigrin, but significantly depleted concentrations of progoitrin, compared to leaf, stem and nymphal tissues (Figure 5.2). The depleted concentration of progoitrin corresponded to an increased proportion of progoitrin in the adult aphids honeydew (Figure 5.3), suggesting that progoitrin was being ingested but was excreted. Flowering stems tended to have lower concentrations of glucosinolates than leaves (this difference was significant for sinigrin, gluconapin, gluconapoleiferin and glucobrassicin; Figure 5.2). Relative to the flowering stem (the feeding site of the aphids), nymphal aphids contained significantly higher concentrations of all five glucosinolates (Figure 5.2). Adult aphids resembled stem tissue in terms of gluconapin and glucobrassicin concentration (Figure 5.2). Adult aphids contained significantly less gluconapin and glucobrassicin than nymphal aphids when comparing both concentrations (Figure 5.2) and composition (Figure 5.3) (Tukey's HSD,  $P < 0.01$ ).

Linear discriminant analysis of the 226 wild plants that contained all identified glucosinolates provided a single discriminant function (LD1) that best separated aphid-infested plants from uninfested plants. LD1 correctly predicted the infestation category of plants in 78% of cases (Fisher's exact test, odds ratio = 3.33,  $n=226$ ,  $P = 0.003$ ). The probability of infestation increased with increasing LD1 (GLM with binary error structure,  $\chi^2_1 = 39.39$ ,  $P < 0.001$ ; figure 5.4). LD1 itself increased with increasing concentrations of gluconapin, with decreasing progoitrin and glucobrassicin, and was little influenced by sinigrin and gluconapoleiferin (Figure 5.4). Hence aphids infested plants producing high concentrations of gluconapin but were less likely to infest plants producing high concentrations of progoitrin and glucobrassicin.

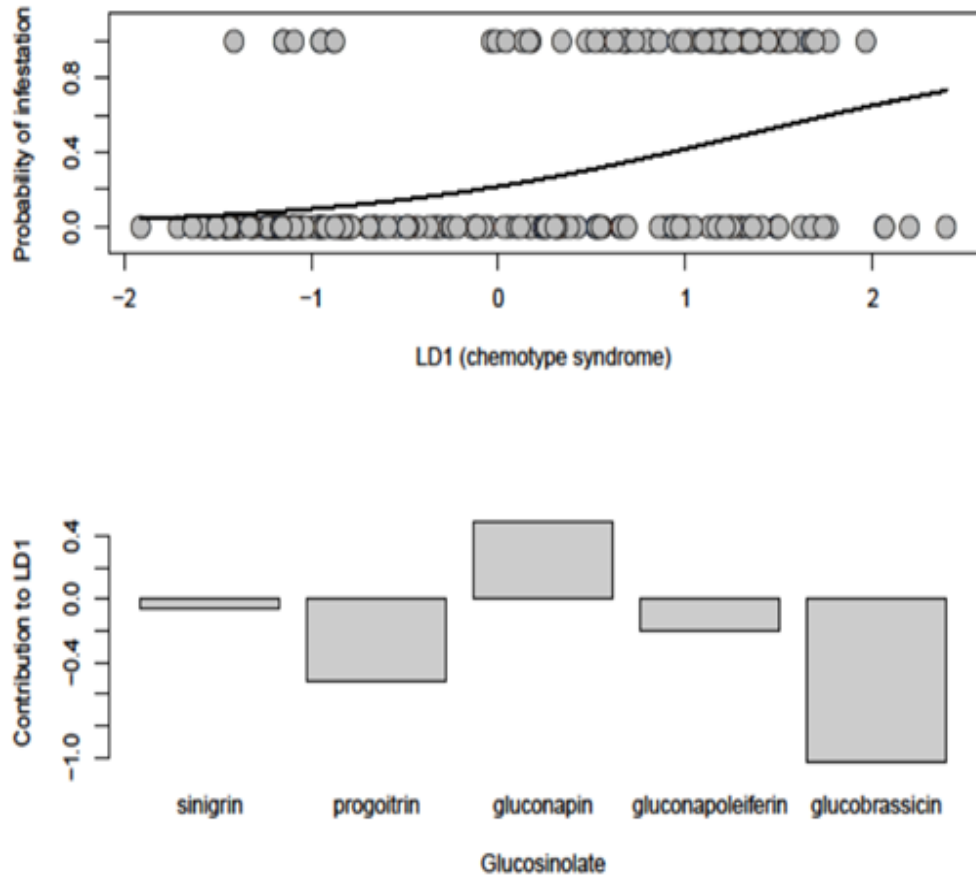


Figure 5.4. **a** Logistic regression of aphid infestation against the glucosinolate profile of 226 wild cabbage plants. The predictor, LD1, is **b** a linear discriminant function with positive loading towards gluconapin concentration, negative loading towards progoitrin and glucobrassicin concentration, with little influence from sinigrin or gluconapoleiferin concentrations

## DISCUSSION

We have shown that a specialist aphid sequesters high concentrations of host-plant chemical defences but does not simply reflect its host plant chemotype. Adult aphids differentially sequester according to both glucosinolate group and structure, and the amount of progoitrin excreted in their honeydew is very high. This pattern of sequestration and excretion maps onto host plant use. Aphids are less likely to infest plants that express high concentrations of progoitrin in their chemotype. Together, these results suggest that this specialist herbivore may not be adapted to all aspects of the Brassica defence system, consistent with a continuing biochemical arms race between plant and aphid.

Several specialist species have been shown to selectively sequester at the level of glucosinolate group. For example, the crucifer flea beetle *Phyllotreta striolata* and the sawfly *Athalia rosae* both exhibit a preference for host plant glucosinolates belonging to the aliphatic group over indoles (Beran et al. 2014; Abdalsamee and Müller 2012). This study reports a similar result. Adult aphids accumulated low concentrations of glucobrassicin, an indole glucosinolate, a finding which is in agreement with the results of Kos et al. (2011). This is likely explained by the fact that indole glucosinolates can be hydrolyzed independently of myrosinase activity (Kim and Jander 2007; Kim et al. 2008), so cannot be stored without causing potential harm to the aphid.

Our results show that wild *B. brassicae* adults also differentially sequester aliphatic glucosinolates. Our finding of sinigrin sequestration and accumulation was expected, considering observations of aphid myrosinase activities (Francis et al. 2002), and the toxicity of sinigrin breakdown products to various aphid natural enemies (Francis et al. 2001; Kazana et al. 2007; Kos et al. 2012). However, we also

observed that the aliphatic glucosinolate progoitrin was stored at very low concentration in aphid tissue, and the proportion excreted in aphid honeydew was very high. Although little is currently known regarding the metabolism of glucosinolates in aphids, this new result does fit with known activities of the aphid's myrosinase enzyme (Francis et al. 2002). While progoitrin belongs to the same aliphatic chemical group as sinigrin, differences in the side chain structure of glucosinolates determine the chemical properties of compounds resulting from myrosinase degradation (Macleod and Rossiter 1986; Mithen 2001).

Glucosinolates are commonly hydrolysed to yield isothiocyanates (Macleod and Rossiter 1986; Rossiter et al. 1990). However, progoitrin exhibits greater thermal instability than glucosinolates such as sinigrin, and due to the 2-hydroxy group in this compound, degradation environment can result in the unique formation of other chemicals such as nitriles and thionamides (Macleod and Rossiter 1986). While the biological effects of these compounds are not known, such characteristics may make progoitrin unsuitable, or potentially harmful, for aphids to accumulate or retain. Furthermore, while aphid myrosinase activity towards sinigrin is high, the rate of hydrolysis of progoitrin is much lower (Francis et al. 2002). Our finding of high progoitrin content in excreted honeydew, coupled with the biochemistry discussed above, suggests that not all aliphatic glucosinolates are beneficial for this specialist aphid. We recommend experiments that establish the differential impacts of these compounds on aphid fitness.

Drawing on knowledge of aphid biochemistry may also explain why aphid nymphs have different patterns of sequestration and excretion than adults. Nymphs accumulated all identified glucosinolates, but not to the same extent as adults. Low accumulation of sinigrin by *B. brassicae* nymphs was also



found by Kazana et al. (2007), and it may be that the ability to target glucosinolate structures develops with aphid life stage. This hypothesis is supported by mechanistic studies investigating the molecular basis for *B. brassicae*'s adaptation to plant glucosinolates, which indicate that expression of myrosinase enzyme increases throughout aphid development (Jones et al. 2001; Kazana et al. 2007). We do not yet know what transport mechanisms are involved in glucosinolate uptake, but postulate that these also develop with aphid life stage. Further work identifying transport mechanisms, and their ontogenetic development, will provide valuable insights into stage-specific physiological costs and constraints of sequestration in this aphid.

Finally, based on the findings of Züst et al. (2012), we predicted patterns of sequestration and excretion would be reflected in host plant use, with aphids preferentially feeding on specific chemotypes high in 3-carbon aliphatic glucosinolates, but avoiding plants expressing high concentrations of 4-carbon glucosinolates. Our results showed that the likelihood of aphid infestation was not influenced by variation in sinigrin concentration, part of the 3-carbon group, but was negatively affected by higher concentrations of the 4-carbon glucosinolate progoitrin, and the indole glucobrassicin. The lack of effect of sinigrin concentration on host plant preference might be due to the ability of *B. brassicae* to concentrate sinigrin efficiently. Studies of other sequestering specialists, such as the monarch butterfly, *Danaus plexippus*, found that larvae were able to concentrate even low levels of cardenolide toxins from their milkweed host plants (Malcolm et al. 1989). Similarly, Kazana et al. (2007) reported that even aphids reared on low sinigrin diets were able to accumulate high concentrations of this compound. Conversely, our finding that aphids are less likely to infest plants high in progoitrin suggests that progoitrin might harm aphids directly, or that excretion of this compound imposes a physiological cost.

However, our surveys of infestation cannot tease apart the influence of aphid preference and aphid performance on different plant chemotypes. Further experimental work using common garden experiments is required.

A result more difficult to interpret is yielded by the aliphatic glucosinolate, gluconapin. Plants expressing high concentrations of gluconapin were more likely to be infested by aphids, even though this chemical is part of the 4-carbon group that is proposed by Züst et al. (2012) to have a negative effect on aphid biomass (Züst et al. 2012). Concentrations of gluconapin were lower in adult aphids than in nymphs, suggesting a similar, albeit weaker sequestration pattern to progoitrin. One key difference between these two glucosinolates is that the expression of gluconapin is produced by the same functional allele that yields sinigrin (*GS-AOP-ALK*) in the 3 carbon biosynthesis pathway (Figure 5.1). Gluconapin is the chemical precursor to progoitrin, but progoitrin requires further modification yielded by the allele, *GS-AOP-OH* (Züst et al. 2012). We suggest that the hydroxylation of glucosinolates, promoted by *GS-AOP-OH*, deters aphid herbivory and may represent an adaptive response by the host plant that prevents the co-option of alkyl glucosinolates by aphids. In order to test the causality of aphid selection on *GS-AOP-OH*, multigenerational selection experiments, rearing aphids on a range of Brassicaceae plant species that vary in their chemotype profiles, are needed.

In summary, we have shown that aphids selectively sequester host plant defences, storing high concentrations of the compound sinigrin but excreting progoitrin in their honeydew. This apparent aversion to progoitrin maps on to patterns of host plant infestation in wild populations. While several studies have demonstrated links between plant defence chemistry and herbivore feeding preferences and performance, nearly all are performed in the laboratory or greenhouse under controlled conditions. Only rarely are these results validated, or even tested, in wild plant populations (but see Baldwin et al. 2001). Sequestration of sinigrin, and excretion of progoitrin, fits well with geographic patterns of infestation and selection pressures on *Arabidopsis* chemotypes (Züst et al. 2012). Experiments testing the effects of sinigrin and progoitrin on aphid fitness, and investigating the response of Brassica plants to aphid infestation, are now required in order to fully understand the ecological significance of differences in glucosinolate sequestration and excretion.

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## **CHAPTER SIX**

### **OPTIMAL SEQUESTRATION OF PLANT DEFENCE CHEMISTRY AMONG APHID MORPHS AND LIFE STAGES**

## ABSTRACT

Optimal defence theory has been widely and successfully applied in explaining patterns of secondary metabolite allocation across somatic and reproductive tissues in plants. Surprisingly, this theory has never been extended as a potential explanation for the distribution of sequestered plant defence compounds in herbivorous insects. In the present study, we use life tables to determine the reproductive value of instars and morphs of the brassica specialist aphid, *Brevicoryne brassicae*. We then analyse variation in sequestered sinigrin, an important aphid defence compound, across instars and morphs. Significantly higher concentrations of sinigrin were observed for the morph of highest reproductive value, the wingless adult, compared to nymphs and winged dispersers which exhibit lower reproductive value due to zero fecundity or high stochastic mortality, respectively. These results are in accordance with optimal defence theory, predicting higher allocation of sequestered sinigrin in the aphid morph most important for the fitness of the clonal genotype. Our results generate further predictions of reduced defence investment in senescent and menopausal aphid mothers (due to low reproductive value), and increased investment among successful winged dispersers (due to high reproductive value post-dispersal).

## INTRODUCTION

Optimal defence theory (ODT) has been fundamental in explaining observed spatial and temporal patterns in anti-herbivore defence chemistry among plant tissues, predicting greater allocation of costly defenses to plant parts that are of highest reproductive value, or most vulnerable to attack (McKey 1979; Rhoades 1979; Zangerl and Rutledge 1996). ODT has proven very successful in clarifying the adaptive value of differing plant defence strategies, yet this predictive framework has rarely been extended to chemical defence allocation in other organisms. This is surprising given that many species of herbivorous insect sequester toxins from their host plant, generating effective defences against their own natural enemies (Nishida and Fukami 1990; Nishida 2002). Furthermore, the distribution of sequestered chemicals in insects has been shown to vary quantitatively and qualitatively, within and between individuals (Nishida 2002 and references therein).

The consequences of this variation have largely been examined from the perspective of costs associated with sequestration. A growing number of studies show that sequestering herbivore species selectively target, and actively accumulate, specific chemicals from within their food plants, and are not simply dependent on host plant variation in chemical content (Kazana et al. 2007; Zagrobelny and Møller 2011; Fürstenberg-Hägg et al. 2014). This requires specific physiological mechanisms and incurs energetic costs associated either with transporting and storing compounds, or through the direct effect of sequestered toxins on the insect (Malcolm and Zalucki 1996). These costs are often reflected in negative correlations between measurements of insect performance (e.g. reduced mass, longer development time, lower fecundity) and the concentration of compounds within the host plant (Bowers and Collinge 1992; Camara 1997; Richards et al. 2012; Mason and Singer 2015).

However, when the central assumption of costs to defense is met, ODT offers an adaptive explanation for variation in sequestered defense, predicting that individuals should allocate defenses in a way that maximizes inclusive fitness (McKey 1974; Rhoades 1979). In the case of insect herbivores, the theory implies that life stages and body parts which have the highest reproductive value, or greatest risk of attack, should have the highest levels of chemical defense. Sequestered chemicals are not uniformly distributed within an insect's body, and concentrations may also vary with age and sex. For example, higher concentrations of defenses have been reported in egg-laying females compared to males in sequestering butterflies and bugs (Brower and Glazier 1975; Scudder et al. 1986; Fordyce and Nice 2008). Similarly, high concentrations are often stored in body parts which are highly vulnerable to predator attack, such as the abdomen and wings in monarch butterflies (Brower and Glazier 1975). Finally, concentrations of sequestered plant compounds vary with age or life stage, and may increase with larval instar, or exhibit significant differences between larval and adult stages (Whitman et al. 1992; Opitz and Müller 2009; Reudler et al. 2015).

In order to fully extend ODT to the allocation of sequestered defences, the contribution of different life stages and/or tissues to an individual's fitness needs to be assessed. Measuring the reproductive value of different tissues or life stages within individuals or assessing their contribution to inclusive fitness is challenging. For a number of reasons, aphids provide desirable model systems. Due to apomictic parthenogenetic reproduction, colonies can be monoclonal (Hodgson 2001), and therefore genetic conflict is absent (but see Lushai and Loxdale 2002 for critique). Thus, aphid colonies can be conceptually thought of as unitary organisms (Hodgson 2001; Pike and Manica 2006) and allocation of natural enemy defences should be selected for at the level of the clonal genotype. Furthermore, polymorphism promotes differential allocation of reproductive and dispersal resources (Powell et al.

2006), whereby females produce nymphs which can either develop into apterous (wingless) females, or alate (winged) females which are able to disperse (Dixon 1977). Detailed studies of life-history differences among aphid morphs have demonstrated trade-offs among these fitness components: across aphid species, winged females are consistently less fecund than wingless adults, take longer to reach reproductive maturation and experience high levels of stochastic mortality during dispersal (Dixon 1977; Wratten 1977; Groeters and Dingle 1989; Dixon and Kindlmann 1999). We can extend the predictive framework of ODT to the aphid's clonal genotype: if natural enemy defences are costly, it is predicted that investment should be highest in the most reproductively valuable lifestages and morphs. In order to test these predictions, we utilize the specialist aphid *Brevicoryne brassicae* as a model system.

*B. brassicae* selectively sequesters the glucosinolate sinigrin from its brassica host plants to high concentrations (Goodey et al. 2015). When the aphid's body is damaged by predation, sinigrin is converted into toxic by-products through the action of an endogenous aphid enzyme (Bridges et al. 2002; Kazana et al. 2007; Pontoppidan et al. 2003). This defence system is considered an exemplar of plant-herbivore coevolution: the insect has evolved its own enzyme to catalyse the breakdown of the sequestered plant compound (Bridges et al. 2002). Multiple studies have reported the direct and indirect defensive properties of sinigrin sequestered by *B. brassicae*. For example, in predator defence, ladybird larvae, *Adalia bipunctata* reared on wingless aphids fed on a high sinigrin diet failed to complete development (Pratt et al. 2008) and the performance of *Episyrphus balteus* hoverfly larvae was lower when fed aphids containing high levels of glucosinolates (Kos et al. 2012), suggesting the ingestion of glucosinolates results in autotoxicity. Sinigrin has also been identified as a component of aphid alarm pheromone, which when released signals threat to the rest of the colony (Bridges et al. 2002).



Here we argue that the observed differential allocation of sequestered sinigrin to different morphs should fit with the assumptions of ODT. Several studies have shown that high sinigrin concentrations in host plants are detrimental to aphid performance (Cole 1997a; Newton et al. 2009a), suggesting that handling sinigrin is costly and therefore that sequestration should be used economically. Specifically, we predict that sequestration of sinigrin will be highest among wingless adult females because they contribute most to clonal fitness and are most vulnerable to attack; low for nymphal aphids because they have low reproductive value and therefore should invest in maturation instead; and low for winged dispersers because stochastic mortality reduces their reproductive value. We validate our predictions using a model of reproductive value of aphid morphs and instars, derived from life tables of laboratory-reared *B. brassicae* (Mirmohammadi et al. 2009).

## MATERIALS AND METHODS

*Modelling reproductive value.* We built a life history projection matrix using life table parameters calculated by Mirmohammadi et al (2009) from *B. brassicae* colonies reared on laboratory cultures of oil seed rape, *Brassica napus*.

The age- and stage- structured matrix was derived from a life cycle graph depicting the probability of surviving between different ages/stages, and the contribution of each stage to fecundity. We present a simplified version of this model in figure 6.1. The model contains lifestages for each day of nymphal development, followed by an equal probability (0.5) of transitioning to wingless or unwinged adult female morphs, each of which passes through several age classes before ending their reproductive lifespan. The matrix elements in the resulting projection matrix (table 6.1) represent transitions between these life stages across the life span of the aphids. Survival probability of all ages of winged adults was assumed to be equal to those of same-aged unwinged adults, with the exception of winged adults in the dispersal stage. Mortality is very high in this stage class, due to low success rates of locating a suitable new host plant (Ward and Leather 2010).

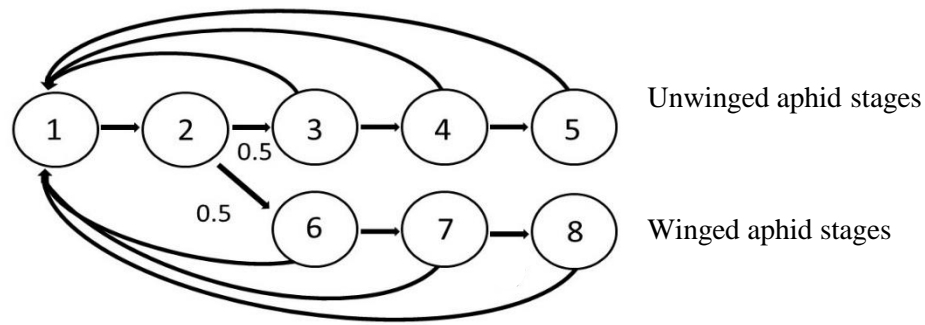


Figure.6.1. Simplified life cycle graph showing possible transitions between eight life stages of the aphid, *Brevicoryne brassicae*. This simplified life cycle has two age classes of nymphs, and three age classes each of unwinged and winged adult females, with an equal probability (0.5) of nymphs transitioning to winged or unwinged life stages. The true age-classified life cycle has many more stages, hence is too large to represent graphically.

Demographic modelling of the projection matrix was conducted using the R package popdemo (Stott et al. 2012). The real life history projection models were of dimension 53x53, because life tables contained 7 days of nymphal development, 13 days of adult wingless reproductive lifespan, and 13 days of adult winged reproductive lifespan (Mirmohammadi et al. 2009). In the absence of explicit data on survival and fecundity of winged aphids, we assumed that they followed the same survival and fecundity schedule as wingless adults once they had settled on a new host plant. We used an arbitrary value for survival of dispersing winged adults ( $d=0.001$ ), but note that all results are completely robust to changes in this mortality risk as long as  $d < 0.5$ .

Table 6.2. Matrix (A) of life stage transitions for a simplified life history model of the aphid, *Brevicoryne brassicae*, where parameter  $l_x$  is the age specific survival probability per time step,  $m_x$  is age specific fecundity, and  $d$  is the probability that a winged disperser survives to find a new plant. Columns of the matrix represent ages at time  $t$ , and rows represent ages at time  $t+1$ . At the end of age 2, half the surviving aphids become unwinged adults and half become winged adults

$$\mathbf{A} = \begin{bmatrix} 0 & 0 & l_3 m_3 & l_4 m_4 & l_5 m_5 & dl_3 m_3 & l_4 m_4 & l_5 m_5 \\ l_1 & 0 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0.5l_2 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & l_3 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & l_4 & 0 & 0 & 0 & 0 \\ 0 & 0.5l_2 & 0 & 0 & 0 & 0 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & dl_3 & 0 & 0 \\ 0 & 0 & 0 & 0 & 0 & 0 & l_4 & 0 \end{bmatrix}.$$

We calculated age- and stage-specific relative reproductive values from the resulting life-history projection matrix, using the dominant left eigenvector of the matrix, scaled to sum to one (Caswell 1982). These reproductive values estimate the value of each life stage to the numerical rate of increase of the clonal genotype, discounted by future mortality risks. A direct link to fitness assumes that the abundance of the genotype is increasing exponentially (Schaffer 1974; Caswell 1982), which is a fair assumption for pest species of aphids, especially at the start of the growing season.

*Collection of aphid life stages.* In May 2014, 40 flowering plants with evidence of aphid infestation were randomly selected from a population in Prussia Cove, Cornwall, UK (SW 55653 28034). The 40 aphid colonies were allowed to develop naturally for four weeks with exposure to natural enemies, after which time, aphid colonies were bagged in organza and transferred to the laboratory. A subset of nymphs, adult winged and wingless aphids (20 individuals of each lifestage where possible) were brushed into sample vials for extraction of sinigrin.

*Extraction and Analysis of Intact Sinigrin.* After harvest, aphid material was immediately frozen in liquid nitrogen, lyophilized to dryness, weighed, and ground to a powder using tungsten beads with a diameter of 2 mm and a vibrating-ball micro mill (based on the Retsch MM300 ball mill). Tissue disruption was carried out for 4 min at a vibration frequency of 25 -1s. Insect material was transferred into 1.5 ml microcentrifuge tubes. Sinigrin extraction was conducted in cold, 80% methanol (Goodey et al 2015) and 200 µl of extraction medium (80% methanol containing internal standards umbelliferone, 7.2 µg/ml, and linamarin 1.25 µg/ml) was added to each sample. Samples were incubated on ice for 30 minutes with vortex mixing every ten minutes, followed by 15 minutes of sonication.

After centrifugation (10 min at 16,100 x g, 4 °C), the supernatant was decanted and filtered through a 0.45 µm (PVDF) syringe filter (Chromacol, Welwyn Garden City, UK).

Quantitative glucosinolate analysis was performed using multiple reaction monitoring (MRM) on an Agilent 6420B triple quadrupole (QQQ) mass spectrometer (Agilent Technologies, Palo Alto, USA), coupled to a 1200 series Rapid Resolution HPLC system. Sample extracts (15 µl) were loaded onto a Zorbax Eclipse Plus C18 3.5 µm, 2.1 x 150 mm reverse phase analytical column (Agilent Technologies, Palo Alto, USA). The following gradient was used: 0 min – 0% B; 1 min – 0% B; 5 min – 20% B; 20 min – 100% B; 25 min – 100% B; 27 min – 0% B. QQQ source conditions were as follows: gas temperature 350°C, drying gas flow rate 9 l min<sup>-1</sup>, nebuliser pressure 35 psig, capillary voltage ±4 kV. The presence of sinigrin was confirmed by comparing retention times and ion fragmentation patterns with pure sinigrin standard (Sigma-Aldrich Chemie GmbH, Deisenhofen, Germany and Merck KGaA, Darmstadt, Germany).

*Statistical Analyses.* Statistical analyses were performed in R 3.1.2 (R Core Team 2014). All analyses were performed using log<sub>10</sub>-transformed sinigrin concentrations: this transformation normalized the residuals and satisfied the homogeneity of variance assumption for standard parametric statistical tests. We compared the concentrations of sinigrin among aphid sample tissues using one-way analysis of variance followed by Tukey's HSD test to compare between tissues without risking Type I errors. Comparisons were made between nymphal aphid, adult wingless aphid and adult winged aphids.

## RESULTS

*Aphid life tables and reproductive value.* When reared in the laboratory, nymphal *B. brassicae* have very high rates of survival (figure 6.2a). Rates of survival then decline rapidly during the aphids' adult lifespan. Reproductive maturity is first reached at age 8 days, and fecundity peaks at age 14, falling to zero at age 32 (figure 6.2b). Aphids can survive beyond this age but no longer contribute directly to clonal fitness (see Uematsu et al. 2010 for discussion of the indirect reproductive value of menopausal aphids). These schedules of survival and reproduction translate to a single-humped age-distribution of reproductive value in unwinged aphids, rising quickly from age 8 days, peaking at age 11 days, then falling rapidly (figure 6.3). Winged aphids suffer a two-humped schedule of reproductive value, caused by high rates of stochastic mortality at age 8 days (timing of dispersal). If these dispersing aphids find new host plants, their reproductive values return to the same values as same-aged wingless morphs because of our assumption of equal mortality schedules (table 6.1).

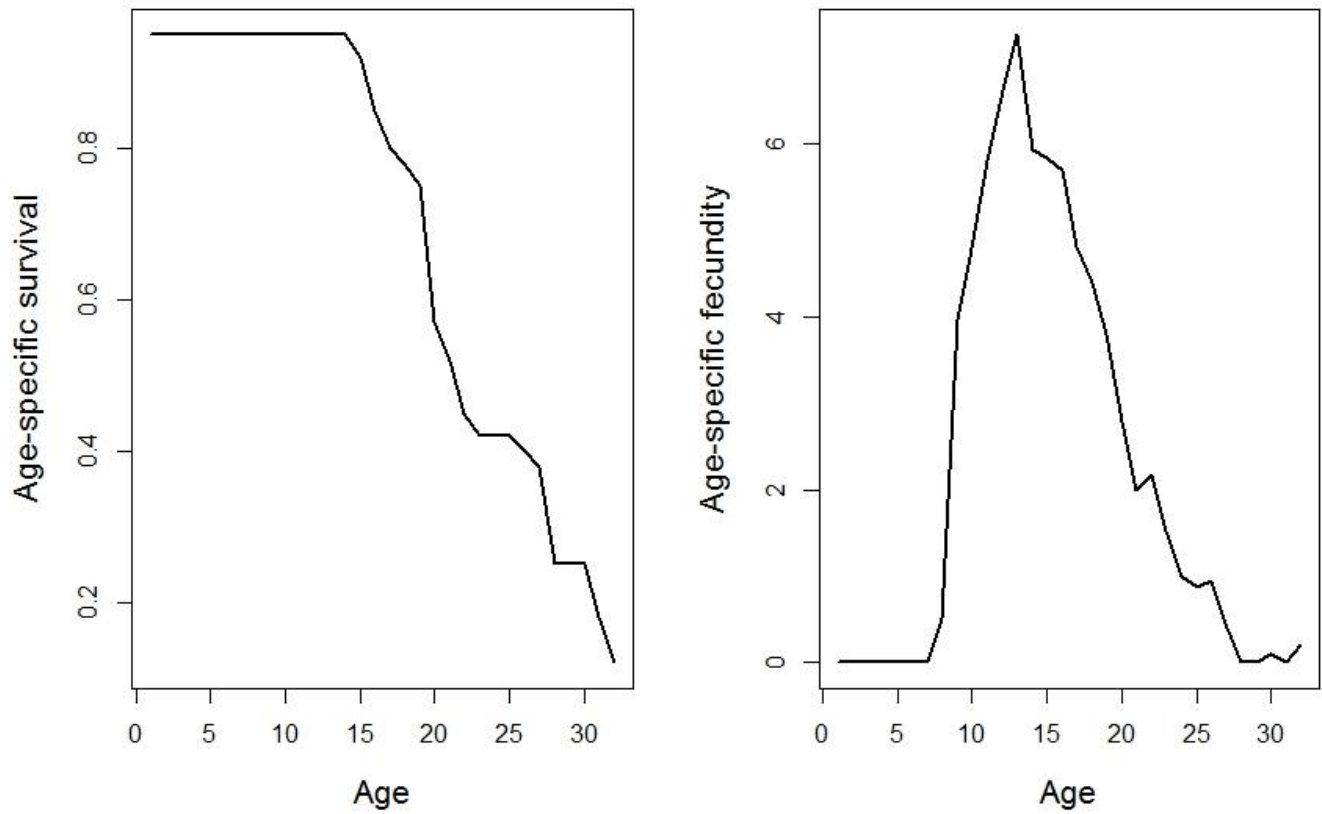


Figure 6.2 a & b. Age specific survival (L) and fecundity (M) in laboratory reared *Brevicoryne brassicae*.

Nymphs initially have high rates of survival. Rates of survival for nymphs are high, then decline rapidly during the aphids' adult lifespan. Reproductive maturity is first reached at age 8 days, and fecundity peaks at age 14 but then falls to zero at age 32.



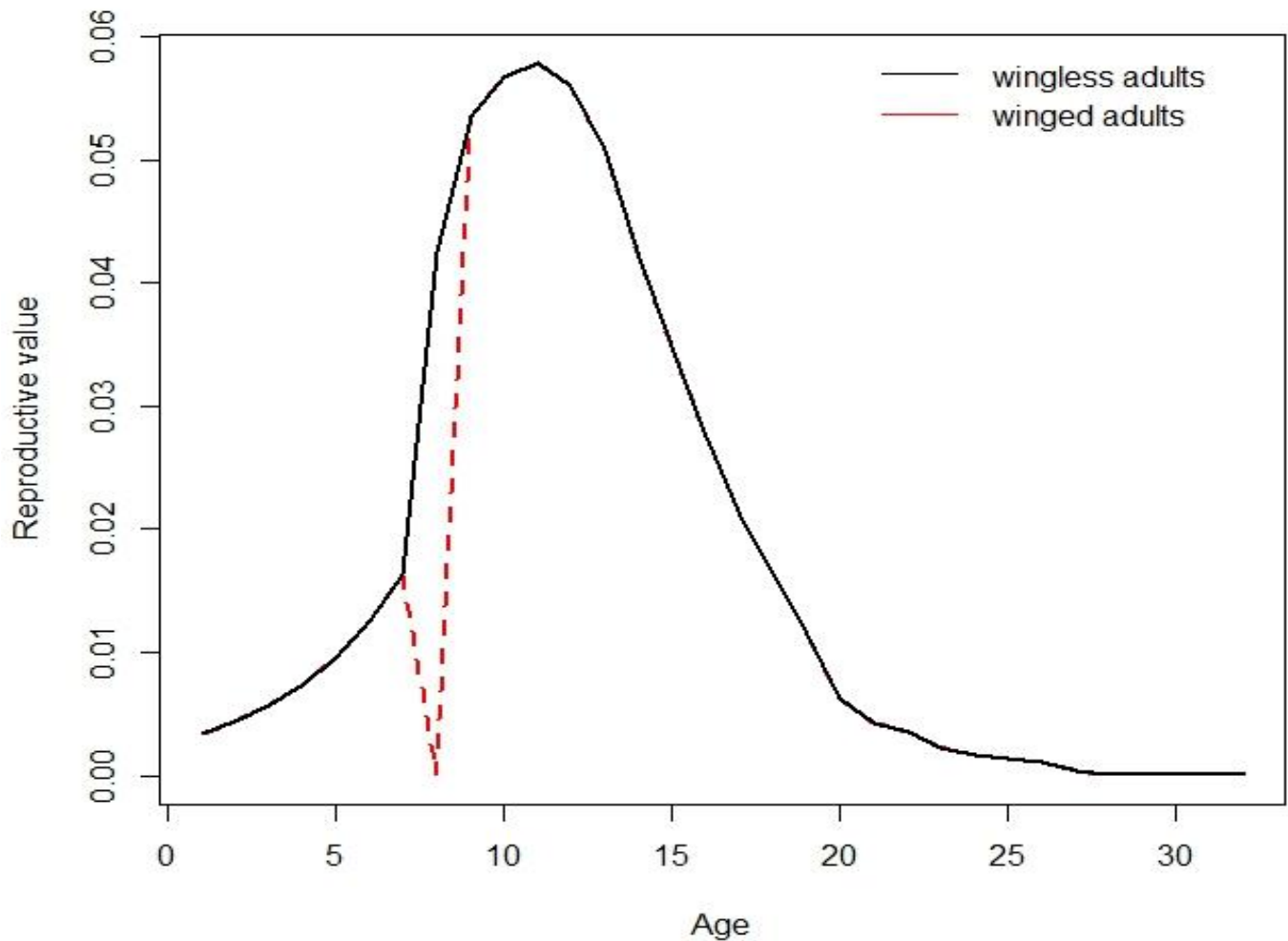


Figure.6.3. Reproductive values of winged and wingless *Brevicoryne brassicae* aphids. Reproductive value calculated from the dominant left eigenvector of a life history projection model. Black line follows the age-schedule of reproductive value of unwinged aphids. Winged aphids (red line) follow the same schedule but suffer a collapse in reproductive value at reproductive maturity, which coincides with the expression of the winged phenotype and the risk of mortality during dispersal. Successful dispersers then exhibit the same schedule of reproductive value as their unwinged counterparts.

*Aphid Sinigrin Concentrations.* Across aphid samples, sinigrin concentrations varied significantly among life stages (one-way ANOVAs comparing relative sinigrin concentrations among nymphal aphid, adult wingless aphid, winged aphids ( $F_{2,169} = 29.46$ ;  $P < 0.001$ ). Tukey's HSD multiple comparisons of means showed significant differences between nymphs and winged adults ( $P < 0.01$ ), and wingless adults contained significantly greater concentrations than both other lifestages ( $P < 0.001$ ) (figure 6.4).

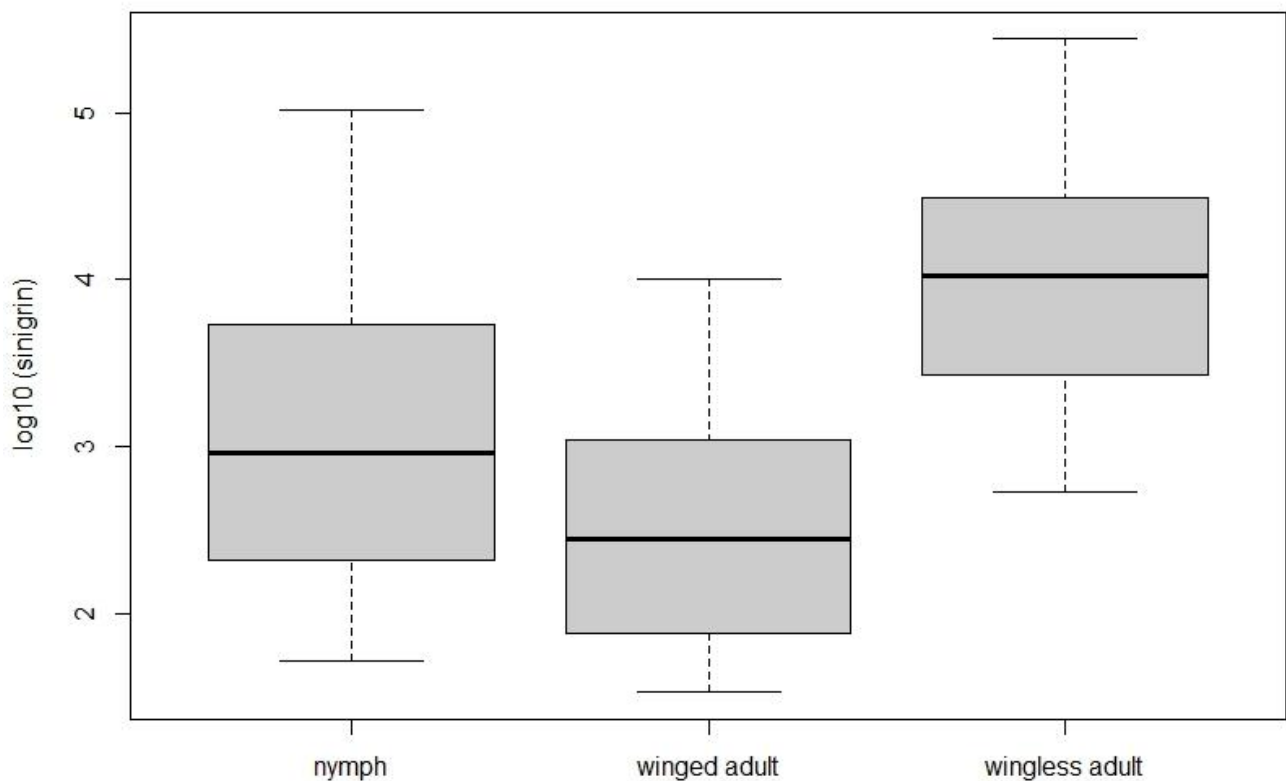


Figure 6.4. Log10 transformed relative concentrations of the glucosinolate sinigrin in matched samples of aphid nymphs, wingless adults and winged adults. Boxplots show range (whiskers), interquartile range (boxes), median (thick line) and putative outliers (open circles).

## DISCUSSION

Our results show that wingless adult females have the highest reproductive value across the *B. brassicae* life cycle, and contain significantly higher concentrations of sequestered sinigrin than winged females or nymphs. These results are congruent with optimal defence theory: aphids apparently invest more of their limited resources in the chemical defence of the most reproductively valuable life stage. Whilst differential sequestration patterns have been reported previously in winged versus wingless aphid morphs (Kazana et al. 2007), this variation was discussed solely in terms of physiological constraints. Here we offer an adaptive explanation and, to our knowledge, the first explicit test of ODT in an insect that sequesters plant chemicals.

ODT predicts that when defences are costly, they should be allocated to plant tissues that contribute most to fitness and that are highly vulnerable or apparent, to attack (McKey 1979). Our model of reproductive value, based on life history tables of *B. brassicae*, showed that wingless adult females contributed most to clonal fitness. In this study we have not tested the vulnerability of different life stages or morphs to attack from natural enemies, yet it is reasonable to assume that wingless aphids, unable to escape predation or parasitism through dispersal, may suffer greater threat than their winged counterparts and must rely on other defence strategies. It is interesting to note that wingless females, the most highly defended morph, survive for around ten days post-reproduction. We could not separate pre- and post-reproductively active females when measuring sinigrin concentrations, but ODT suggests that low reproductive value should result in lower investment in defence. Considering a recent study, which found an adaptive role for menopausal aphids in terms of altruistic defence (Foster 2010), we suggest that detailed study of defensive sequestration could reveal exciting changes in post-reproductive lifestages.

Based on their lower reproductive value due to a high risk of stochastic mortality, we predicted that winged aphids would be less chemically defended. However, our estimates of differences in reproductive value between winged and wingless females may actually be conservative: studies on other aphid species show that wingless females have shorter development times and carry more embryos than winged females, resulting in a reproductive output 70% greater than that of winged morphs (Wratten 1977; Leather et al. 1988). This could mean that reduced investment in defence in winged aphids is due not just to mortality during dispersal, but also to relatively low rates of fecundity in this morph.

Physiological trade-offs between defence and dispersal may exist for winged aphid morphs. During the final days of development, winged aphids rapidly increase their lipid stores (Liquido and Irwin 1986). This increased energetic investment in preparing for flight has been proposed as an explanation for the differential sinigrin sequestration between winged and wingless morphs observed in a previous laboratory study (Kazana et al. 2007). Aphid myrosinase was found to be localized in head and thoracic tissue (Bridges et al. 2002). As the thoracic muscle develops along with wing formation, it may be that winged aphids lose the capacity to store adequate amounts of myrosinase to catalyse sinigrin, therefore accumulating high concentrations of this compound would be counterproductive. On successful location of a new host plant however, the wings of dispersers are autolyzed. At this point our model predicted that the reproductive output of these aphids increases to match the output of wingless females. Although, as previously mentioned, differences in wingless and winged morph fecundity may remain post-dispersal, we still predict that sinigrin sequestration should be upregulated as the winged aphid once more switches tactics to optimize its defence and recommend further work characterizing sinigrin sequestration patterns at multiple developmental points within *B.brassicae*'s life cycle.

Patterns of sinigrin concentration among aphid morphs also revealed low sinigrin concentrations in nymphal aphids. Lower defence allocation in nymphs fits with the low reproductive value of this life stage revealed by our model. Developing aphid embryos possess both myrosinase and sinigrin, however, enzyme expression and sinigrin concentration increase throughout nymphal development (Kazana et al. 2007). This may indicate a trade-off between aphid growth and defence; a widely accepted phenomenon in studies of plant defence allocation (Buckley and Avila-Sakar 2013).

In summary, our results indicate that patterns of sequestered sinigrin fit with optimal defence theory. By extending the framework of ODT to a sequestering insect herbivore, we have provided explanations for why certain life stages and morphs are more heavily defended than others. Wingless adult aphids are heavily defended, which we explain as being due to their high reproductive value. Nymphal aphids are poorly defended, which we suggest is due to their low reproductive value, and their need to allocate limited resources to rapid maturation. Winged adults suffer immediate reduction in reproductive value due to the risk of mortality during dispersal. Overall, we suggest that age- and stage-specific patterns of defence allocation can be explained in an adaptive framework, rather than the more conventional constraints framework. The adaptive framework yields further predictions, including that successful winged dispersers should increase their levels of defence, while menopausal adult aphids should either massively increase their defence or remove it in favour of alternative forms of colony defence. We recommend further study of the direct fitness consequences of sinigrin sequestration, coupled with in depth analysis of the ontogeny of this ability, to help clarify adaptive explanations for aphid defence strategies. We also recommend the use of the reproductive value framework for study of schedules of chemical defence in the life histories of other herbivore species, both generalist and specialist.

## CHAPTER SEVEN

### DISCUSSION

*'...the plant-herbivore 'interface' may be the major zone of interaction responsible for generating terrestrial organic diversity.'*

*Ehrlich & Raven (1964)*

It is now widely accepted that many of the plant secondary metabolites identified so far (alkaloids, cyanogenic glycosides, glucosinolates, terpenes, tannins and many more, Wink 2003) represent adaptive traits that have diversified in response to natural selection in order to defend these sessile organisms from myriad biotic attackers (Wink 2003). However, at a micro-evolutionary level there remains debate regarding the nature of selection exerted by insect herbivores on plant secondary profiles, hindered in part by the tendency to study herbivore responses to single compounds under controlled conditions (**chapter one**). This is a classic challenge for the ecological validity of secondary metabolite-plant-herbivore interactions described in laboratory settings: Are the interactions real and ecologically relevant?

In this work, each chapter has been written as a separate piece of research intended for publication. Yet all chapters are also constituents of a common goal aimed towards describing the complexity of plant secondary phenotypes and investigating the responses of herbivores to this complexity in natural systems. I have illustrated the multivariate nature of plant defence phenotypes, seemingly unconstrained by trade-offs and displaying interactions across a suite of chemicals from different

biosynthetic pathways. This mixture has the potential to shape patterns of herbivore abundance and host plant utilization differentially in natural plant populations; therefore there is the potential for selection on plant chemotype through variable herbivore preferences and aversions. In addition, variation in the concentrations of particular compounds appears to have consequences for specialist counter adaptations: in order to optimally defend against their own natural enemies, aphids sequestering glucosinolates from their host plants must do so selectively, and must choose plants whose chemical profile best matches this behaviour. Together these results highlight the complexity inherent in plant-insect interactions, the importance of field studies and generate a wealth of testable hypotheses for future work, which will be the topic of this discussion.

*Living in a multivariate world, and I am a multivariate...plant*

The main aim of this thesis was to identify the multivariate nature of plant defence profiles, describe the magnitude of variation in phenotypes across ecological scales, and to investigate herbivore responses to this variation. The first step towards this goal was to identify as much of a plant's glucosinolate profile as possible, and this was achieved through the development of a cold extraction technique and rapid quantification using liquid-chromatography-mass-spectrometry (**chapter two**). Unfortunately we were not able to identify aromatic glucosinolates, and defence phenotypes include a natural diversity of these compounds which have been shown to play an important role in oviposition behavior of pierid butterflies (Textor and Gershenzon 2009; Agerbirk et al. 2010). However, we were able to quantify ten glucosinolate compounds from two of the three chemical classes present in *Brassicaceae*: indole and aliphatic glucosinolates. Having identified a large number of glucosinolate constituents present in wild *B. oleracea* plants, I described patterns of

variation across multiple populations in the UK, and investigated for evidence of trade-offs between compounds within and between biosynthetic pathways (**chapter two**). I found evidence that variation in wild glucosinolate phenotypes was driven, in the main, by changing concentrations of sinigrin and progoitrin, a result that supports the findings of Newton et al (2009, 2010). Furthermore, instead of finding the predicted negative associations between concentrations of different glucosinolates (Coley et al. 1985), we found positive relationships indicating that the expression of multiple defences might not be constrained for this species. This may indicate genetic linkage, or the linkage of biosynthetic reaction sequences (White 1983; Zangerl and Berenbaum 1990), and has implications for models of plant defence, as these are often contextualized within the framework of costs (Coley et al. 1985; Bazzaz et al. 1987b; Herms and Mattson 1992). If, however, plants are ‘jacks-of-all-trades, masters of all’ (Koricheva et al. 2004), and can produce several defence types without paying considerable trade-offs, then there is the possibility that environmental variability and positive interactions among defenses may make combinations of defenses both possible, and evolutionarily advantageous (Koricheva et al. 2004). Considering the genetic tools now at our disposal, particularly for Brassica species (Baldwin et al. 2001), future work manipulating the ability of plants to express multiple compounds, and testing the consequences of this for defence against herbivory, is both possible and of considerable interest.

These results beg the question: if plants are not physiologically constrained, then what mechanisms are preventing plants from reaching an ‘optimal’ defence phenotype?



*Nowhere to run, nowhere to hide*

This is the ecological dilemma faced by all vascular plant. The expression of secondary metabolites may provide effective defence against some generalist herbivores, but there are no absolute barriers to herbivory (Stotz et al. 1999). Many herbivore species, including generalists, have evolved an array of strategies for circumnavigating or even exploiting the defence system of their hosts (Winde and Wittstock 2011). In **chapter three**, I investigated the responses of specialists and generalists to the glucosinolate phenotypes I had identified and described in wild populations of *B. oleracea* (chapters two and three). Whilst I was unable to partition glucosinolate preferences by diet breadth due to sampling effort, I did find species-specific responses to chemical profiles, and specialist herbivores (*Pieris rapae*, *Pieris brassicae* and *Brevicoryne brassicae*) over-lapped in their preference for high concentrations of progoitrin. One of the main issues with sampling plants once during a season however, is not being able to tease apart plant response to herbivory from herbivore preference for plant chemotype.

The results of chapter four demonstrate that inter-population variation in glucosinolates has the potential to structure the herbivore community, however the mechanisms by which glucosinolates were affecting herbivores were not clear at this scale. I therefore decided to focus on two specialist herbivores and carry out more intensive studies on a finer scale, using optimization theoretical frameworks to generate testable predictions (Parker and Smith 1990).

### *Does mother know best?*

First, I investigated the role of glucosinolates in mediating the relationship between oviposition choice and offspring performance for the large white butterfly, *Pieris brassicae* (**chapter four**). I formulated predictions within the preference-performance hypothesis, a useful construct which assumes a tight, positive correlation between a mothers choice of food-plant and the performance of her offspring (Jaenike 1978; Bossart 2003). Given the importance of glucosinolates for stimulating oviposition and the tarsal receptors of *Pieris* butterflies, I predicted that females would use these compounds to assess the best quality host on which to lay their eggs. However, whilst I found chemotype preferences for egg-laying females both within, and between populations of *B. oleracea*, I found no relationship between these preferences and the performance of developing offspring. Instead I found that in a common garden population, offspring survived better in a common garden population, when reared on plants descended from their own population, suggesting other plant traits may be important selective agents for this species at a local scale.

### *The aphid and the mustard bomb*

Various specialist Brassica herbivores, including *B. brassicae*, have evolved to co-opt the glucosinolate system for their own defence (Winde and Wittstock 2011). This yields the hypothesis that Brassicas themselves must innovate biochemically to adapt to the feeding specialisms of these coevolving herbivores. In **chapter five**, I provided evidence that sequestering aphids do not simply reflect the chemotype of their host plant, but rather selectively accumulate specific compounds and excrete others in their honeydew. Furthermore, the excreted compound, progoitrin, is part of a

derived, 4 carbon pathway disfavoured by aphids, suggesting only the most evolutionarily derived defences are effective against these highly specialized herbivores.

I decided to explore this fascinating result further by once more exploiting optimality theory. This time, I extended the framework of optimal defence theory, commonly used to explain patterns of defence allocation in plants, to predict that the most reproductively important life stage should be the most heavily defended (**chapter six**). My finding that highly fecund, wingless females sequestered the most sinigrin suggests that age- and stage-specific patterns of defence allocation can be explained in an adaptive framework, rather than the more conventional constraints framework. In future experiments, I wish to test this theory by studying the direct fitness consequences of sinigrin sequestration, coupled with in depth analysis of the ontogeny of this ability.

### *Facing the Future*

These results confirm that chemical interactions are taking place in the wild, and together with previous findings I can conclude with considerable confidence that: natural populations of *B. oleracea* exhibit significant variation in secondary profiles (**chapter two**; Mithen et al. 1995b; Moyes et al. 2000; Newton et al. 2009; Poelman et al. 2009); there is the potential for herbivores to exhibit species-specific responses to variation in secondary profiles (**chapter three**; Moyes et al. 2000; Lankau 2007; Newton et al. 2009; Müller et al. 2010; Newton et al. 2010); herbivore pressure fluctuates through time and space (Haukioja 1980; Newton et al. 2010); and that variation in plant secondary chemistry may have consequences for various aspects of herbivore behavior and performance (**chapters four-six**; Renwick and Radke 1988; Roessingh et al. 1992; Smallegange et

al. 2007; Kos et al. 2011; Abdalsamee and Müller 2012). What is now required is a change from the piecemeal study of individual herbivores and individual (or total) glucosinolates, to a systematic assessment of the fitness costs associated with glucosinolates in both plants and herbivores. This is now possible because I have established a rapid and reproducible method capable of measuring a suite of aliphatic and indole glucosinolates expressed in *Brassica oleracea*. Over the next few years of my research career therefore, I plan to determine the importance of each herbivore species for plant fitness, and correlate this index against the impacts of basic and derived glucosinolates on herbivore fitness. Considering the results from this thesis, I expect that specialist herbivores will only be negatively affected by the most derived chemical defences. This would provide fundamental evidence for the link between coevolution and chemical innovation in a plant-herbivore system.

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## APPENDICES

## **APPENDIX 1**

### **CHAPTER THREE: AIC MODEL SELECTION TABLES INVESTIGATING PATTERNS OF HOST PLANT UTILIZATION IN WILD POPULATIONS OF BRASSICA OLERACEA**

Table 8.1. Competing models within  $\Delta 7$  AICc units of the top model, investigating the factors affecting preference of *Cornu aspersum* for *Brassica oleracea* plants in wild populations in Dorset, ranked by AICc.

Intercept	LD1	Exposure	Rosette	Purpleness	Toughness	logLik	AICc	delta	weight
<b>-0.7479</b>	0.5563		0.4131			-70.464	149.3	0	0.159
<b>-1.7</b>	0.5557		0.4825		0.3441	-69.441	149.4	0.13	0.148
<b>-0.7156</b>	0.5697					-71.96	150.1	0.85	0.104
<b>-1.167</b>	0.558		0.4372	0.2491		-70.01	150.5	1.27	0.084
<b>-1.409</b>	0.5695				0.2534	-71.319	151	1.71	0.067
<b>-0.7875</b>	0.5577	0.02109	0.414			-70.462	151.5	2.17	0.053
<b>-1.716</b>	0.5556		0.4824	0.0625	0.3121	-69.421	151.6	2.31	0.05
<b>-1.661</b>	0.5543	-0.02187	0.4817		0.345	-69.439	151.6	2.35	0.049
<b>-1.048</b>	0.5703			0.1998		-71.646	151.6	2.36	0.049
<b>-0.6973</b>	0.5692	-0.0098				-71.96	152.3	2.99	0.036
<b>-1.123</b>	0.5565	-0.02592	0.4362	0.2517		-70.008	152.8	3.48	0.028
<b>-1.428</b>	0.5687			0.05871	0.2245	-71.3	153.1	3.85	0.023
<b>-1.32</b>	0.5674	-0.05136			0.2558	-71.308	153.1	3.87	0.023
<b>-1.765</b>			0.4902		0.3738	-72.609	153.6	4.29	0.019
<b>-0.7284</b>			0.4189			-73.76	153.7	4.45	0.017
<b>-0.9587</b>	0.5683	-0.05259		0.205		-71.635	153.8	4.52	0.017
<b>-1.665</b>	0.5537	-0.02929	0.4813	0.06503	0.312	-69.417	153.8	4.56	0.016
<b>-0.6994</b>						-75.363	154.8	5.55	0.01
<b>-1.176</b>			0.4419	0.2654		-73.259	154.9	5.59	0.01
<b>-1.327</b>	0.5663	-0.05865		0.06354	0.2248	-71.286	155.3	6.04	0.008
<b>-1.528</b>					0.3025	-74.555	155.3	6.04	0.008

<b>-1.793</b>			0.4895	0.07899	0.3361	- 72.576	155.7	6.4	0.006
<b>-1.632</b>		-0.07471	0.4888		0.3762	- 72.586	155.7	6.42	0.006
<b>-0.6541</b>		-0.03987	0.4181			- 73.753	155.9	6.58	0.006
<b>-1.097</b>				0.2385		-74.95	156.1	6.83	0.005

Table 8.2. Competing models within  $\Delta 7$  AICc units of the top model, investigating the factors affecting preference of *Brevicoryne brassicae* for *Brassica oleracea* plants in wild populations in Dorset, ranked by AICc.

Intercept	LD1	Exposure	Rosette	Purpleness	Toughness	logLik	AICc	delta	weight
<b>-0.8255</b>	0.9119		0.6876	-1.021		-37.187	84.9	0	0.24
<b>-1.346</b>	0.9131	0.3221	0.6805	-1.074		-36.954	86.7	1.75	0.1
<b>-0.8788</b>	0.9153		0.6875	-1.048	0.03624	-37.183	87.1	2.21	0.079
<b>-2.31</b>	0.791		0.6748			-39.511	87.4	2.47	0.07
<b>-0.937</b>			0.6345	-1.008		-39.515	87.4	2.48	0.069
<b>-0.888</b>	0.7928			-0.8701		-39.906	88.2	3.26	0.047
<b>-1.294</b>	0.7901		0.6972		-0.4049	-38.853	88.2	3.33	0.045
<b>-2.459</b>			0.6034			-41.336	88.9	3.98	0.033
<b>-1.339</b>	0.9126	0.3232	0.6805	-1.07	-0.00608	-36.954	88.9	4.01	0.032
<b>-1.03</b>				-0.8851		-41.448	89.1	4.2	0.029
<b>-0.7247</b>			0.6411	-0.9407	-0.1222	-39.477	89.5	4.58	0.024
<b>-2.423</b>	0.8102	0.06731	0.6765			-39.503	89.5	4.63	0.024
<b>-1.034</b>		0.06383	0.6294	-1.02		-39.508	89.5	4.64	0.024
<b>-2.263</b>	0.6519					-41.695	89.6	4.7	0.023
<b>-1.463</b>	0.9159	0.3889		-0.9493		-39.601	89.7	4.83	0.021
<b>-1.323</b>			0.6491		-0.4377	-40.756	89.9	4.96	0.02
<b>-2.416</b>						-42.959	90	5.12	0.019
<b>-1.711</b>	0.9081	0.2902	0.6984		-0.4337	-38.753	90.2	5.35	0.017
<b>-0.9439</b>	0.8004			-0.8944	0.03569	-39.902	90.3	5.43	0.016
<b>-2.251</b>		-0.1166	0.613			-41.31	91	6.07	0.012
<b>-1.398</b>	0.6906				-0.3162	-41.326	91	6.1	0.011
<b>-1.313</b>		0.1834		-0.9243		-41.379	91.1	6.21	0.011
<b>-0.9932</b>				-0.8753	-0.01931	-41.447	91.2	6.34	0.01
<b>-1.599</b>					-0.2979	-42.686	91.6	6.68	0.009
<b>-0.8312</b>		0.07527	0.6354	-0.9522	-0.127	-39.466	91.7	6.78	0.008
<b>-2.393</b>	0.6616	0.07324				-41.684	91.7	6.82	0.008

Table.8.3. Competing models within  $\Delta 7$  AICc units of the top model, investigating the factors affecting preference of *Pieris brassicae* for *Brassica oleracea* plants in wild populations in Dorset, ranked by AICc. Presence of *P. rapae* eggs (+) is included as a covariate.

Intercept	LD1	Exposure	Rosette	<i>Pieris rapae</i>	Purpleness	Toughness	logLik	AICc	delta	weight
<b>0.07053</b>	0.5203						-79.217	164.6	0	0.1630
<b>0.6219</b>	0.5392					-0.2041	-78.66	165.7	1.03	0.0980
<b>-0.03968</b>	0.5171			+			-78.924	166.2	1.56	0.0750
<b>0.0729</b>	0.5167		0.1323				-78.97	166.3	1.65	0.0720
<b>-0.2391</b>	0.5159	0.1671					-79.07	166.5	1.85	0.0650
<b>0.2333</b>	0.5289				-0.09865		-79.121	166.6	1.95	0.0620
<b>0.2854</b>	0.5336	0.2001				-0.2166	-78.453	167.4	2.79	0.0400
<b>0.5875</b>	0.5356		0.1105			-0.1907	-78.491	167.5	2.87	0.0390
<b>0.4754</b>	0.5344			+		-0.1728	-78.58	167.7	3.05	0.0360
<b>0.6038</b>	0.5366				0.06867	-0.2394	-78.63	167.8	3.15	0.0340
<b>-0.03925</b>	0.5133		0.1354	+			-78.667	167.9	3.22	0.0330
<b>-0.3903</b>	0.5121	0.1857		+			-78.745	168	3.38	0.0300
<b>-0.2419</b>	0.5113	0.17	0.1343				-78.819	168.2	3.52	0.0280
<b>0.2182</b>	0.5249		0.1267		-0.08808		-78.895	168.3	3.68	0.0260
<b>0.04879</b>	0.5214			+	-0.04763		-78.904	168.3	3.69	0.0260
<b>-0.0846</b>	0.5253	0.1874			-0.1164		-78.939	168.4	3.76	0.0250
<b>0.2499</b>	0.5294	0.2008	0.1111			-0.2033	-78.283	169.3	4.67	0.0160
<b>0.1187</b>	0.5287	0.2056		+		-0.1834	-78.363	169.5	4.83	0.0150
<b>0.4234</b>	0.53		0.1165	+		-0.1554	-78.394	169.5	4.89	0.0140
<b>0.2804</b>	0.5317	0.195			0.05248	-0.2434	-78.436	169.6	4.98	0.0140

<b>0.569</b>	0.533		0.1106		0.06874	-0.226	-	169.7	5.03	0.0130
							78.461			
<b>-0.3963</b>	0.5072	0.1892	0.1377	+			-	169.7	5.07	0.0130
							78.481			
<b>0.4377</b>	0.5308			+	0.08542	-0.2135	-	169.8	5.17	0.0120
							78.536			
<b>0.02331</b>	0.5166		0.1331	+	-0.03368		-	170.1	5.42	0.0110
							78.657			
<b>-0.2882</b>	0.5176	0.1948		+	-0.0642		-	170.2	5.52	0.0100
							78.709			
<b>-0.1007</b>	0.5204	0.1881	0.1276		-0.1059		-	170.2	5.53	0.0100
							78.712			
<b>0.06669</b>							-	170.3	5.68	0.0100
							83.111			
<b>0.06423</b>	0.5236	0.2071	0.1175	+		-0.166	-	171.3	6.71	0.0060
							78.175			
<b>0.2446</b>	0.5275	0.1957	0.1111		0.05229	-0.2299	-	171.5	6.89	0.0050
							78.267			

Table 8.4. Competing models within  $\Delta 7$  AICc units of the top model, investigating the factors affecting preference of *Pieris rapae* for *Brassica oleracea* plants in wild populations in Dorset, ranked by AICc.

Presence of *P. rapae* eggs (+) is included as a covariate

Intercept	LD1	Exposure	Rosette	<i>Pieris brassicae</i>	Purplenes	Toughness	logLik	AICc	delta	weight
<b>-0.8623</b>	0.7707		-0.6231				-55.375	119.1	0	0.105
<b>-0.05679</b>	0.8242		-0.6475		-0.4983		-54.611	119.7	0.65	0.076
<b>-1.179</b>	0.766		-0.6961	+			-54.649	119.8	0.73	0.073
<b>0.2103</b>	0.7443		-0.6717			-0.4004	-54.756	120	0.94	0.066
<b>-0.001577</b>	0.8226	-0.4699	-0.638				-54.825	120.2	1.08	0.061
<b>-0.4101</b>	0.8141		-0.7106	+	-0.457		-54.007	120.8	1.66	0.046
<b>-0.3307</b>	0.8093	-0.4602	-0.7036	+			-54.12	121	1.89	0.041
<b>-0.843</b>	0.7161						-57.404	121	1.92	0.04
<b>0.6539</b>	0.8653	-0.4164	-0.6624		-0.463		-54.166	121.1	1.98	0.039
<b>-0.2059</b>	0.7444		-0.7298	+		-0.3502	-54.182	121.1	2.01	0.039
<b>0.4661</b>	0.7949		-0.6776		-0.3852	-0.2631	-54.383	121.5	2.41	0.032
<b>0.7781</b>	0.7885	-0.3902	-0.6714			-0.3418	-54.387	121.5	2.42	0.031



<b>-0.1194</b>	0.753 8				-0.4472		-56.72	121. 8	2.69	0.027
<b>0.02079</b>	0.758 9	-0.4789					- 56.80 1	122	2.85	0.025
<b>0.2871</b>	0.848 3	-0.4084	- 0.7178	+	-0.4199		- 53.58 2	122. 2	3.07	0.023
<b>0.03766</b>	0.687 1					-0.3309	- 56.91 9	122. 2	3.09	0.022
<b>-1.074</b>	0.702 7			+			- 56.98 7	122. 3	3.22	0.021
<b>0.348</b>	0.783 2	-0.3891	- 0.7237	+		-0.2875	- 53.81 9	122. 6	3.54	0.018
<b>0.03856</b>	0.791		-0.731	+	-0.3622	-0.2182	- 53.85 3	122. 7	3.61	0.017
<b>0.5657</b>	0.785 2	-0.4189			-0.4015		- 56.25 2	123	3.93	0.015
<b>1.013</b>	0.836 8	-0.3798	- 0.6815		-0.3768	-0.209	- 54.02 5	123	3.95	0.015
<b>-0.2117</b>	0.739 4	-0.4788		+			- 56.38 3	123. 3	4.19	0.013
<b>-0.3733</b>	0.736 6			+	-0.4132		- 56.40 5	123. 3	4.24	0.013
<b>0.6722</b>	0.725 5	-0.4212				-0.2808	-56.46	123. 4	4.35	0.012
<b>-0.8253</b>			- 0.5264				- 58.67 5	123. 6	4.46	0.011
<b>0.2469</b>	0.728 2				-0.3524	-0.195	- 56.58 2	123. 7	4.59	0.011
<b>-0.2629</b>	0.678 9			+		-0.2928	- 56.61 3	123. 8	4.65	0.01
<b>0.4193</b>			- 0.5831			-0.4609	- 57.81	124	4.87	0.009

							2			
<b>-1.164</b>			- 0.5908	+			- 57.87 6	124. 1	5	0.009
<b>0.5759</b>	0.828	-0.3792	-0.729	+	-0.3536	-0.1614	-53.5	124. 3	5.2	0.008
<b>0.3138</b>	0.764 6	-0.4235		+	-0.3665		- 55.92 8	124. 6	5.5	0.007
<b>-0.8094</b>							- 60.29 6	124. 7	5.6	0.006
<b>-0.01681</b>			- 0.6326	+		-0.4075	- 57.21 2	125	5.85	0.006
<b>0.3718</b>	0.714 3	-0.429		+		-0.2404	- 56.13 7	125	5.92	0.005
<b>-0.3051</b>			- 0.5327		-0.3202		- 58.33 9	125	5.93	0.005
<b>0.8289</b>	0.761 6	-0.3987			-0.3278	-0.1559	- 56.16 4	125. 1	5.97	0.005
<b>-0.257</b>		-0.3043	- 0.5178				- 58.42 6	125. 2	6.1	0.005
<b>-0.05293</b>	0.716 5			+	-0.3342	-0.1635	- 56.30 9	125. 4	6.26	0.005
<b>0.2518</b>						-0.3955	- 59.57 9	125. 4	6.27	0.005
<b>-0.5541</b>		-0.3324	- 0.5817	+			- 57.57 9	125. 7	6.59	0.004
<b>-1.08</b>				+			- <b>59.75</b> 2	125. 7	6.61	0.004
<b>-0.6774</b>			- 0.5924	+	-0.2897		-57.6	125. 7	6.63	0.004
<b>0.7814</b>		-0.2304	- 0.5687			-0.4326	- 57.67 2	125. 9	6.77	0.004
<b>0.5183</b>			-		-0.1446	-0.4103	-	126	6.94	0.003

			0.5816				57.75 6			
<b>-0.1402</b>		-0.3637					- 59.92 8	126. 1	6.97	0.003

## APPENDIX 2

### CHAPTER FIVE: COMPARISON OF GLUCOSINOLATE EXTRACTION METHODOLOGIES

*Comparison of Glucosinolate Extraction Methods.* After harvest, plant material was immediately frozen in liquid nitrogen and lyophilized to dryness. Leaf material (10mg) from 50 randomly selected plants was combined and ground to a powder using tungsten beads with a diameter of 2 mm and a vibrating-ball micro mill (based on the Retsch MM300 ball mill). 10mg aliquots of combined leaf tissue were transferred into 24 1.5 ml microcentrifuge tubes, and 12 samples were randomly assigned to one of two extraction methods: Hot extraction in 70% methanol or cold extraction in 80% methanol. Tissue disruption was carried out for 4 min at a vibration frequency of  $25\text{ s}^{-1}$ . 400  $\mu\text{l}$  of extraction medium (80% or 70% methanol containing internal standards umbelliferone, 7.2  $\mu\text{g/ml}$ , and linamarin 1.25  $\mu\text{g/ml}$ ) was added to plant samples. Glucosinolate analysis was conducted as stated in Material and Methods.

We compared peak abundances across five identified glucosinolates using paired t-tests. No difference was found between hot and cold treatments for sinigrin;  $t = -0.65$ ,  $df = 11$ ,  $P = 0.53$ ; progoitrin;  $t = 1.50$ ,  $df = 11$ ,  $P = 0.16$ ; gluconapin;  $t = 1.59$ ,  $df = 11$ ,  $P = 0.14$ ; or gluconapoleiferin;  $t = 1.537$ ,  $df = 11$ ,  $P = 0.15$ . However, peak abundances for glucobrassicin were significantly higher in the cold extraction treatment ( $t = 2.57$ ,  $df = 11$ ,  $P = 0.02$ , figure 9.1).

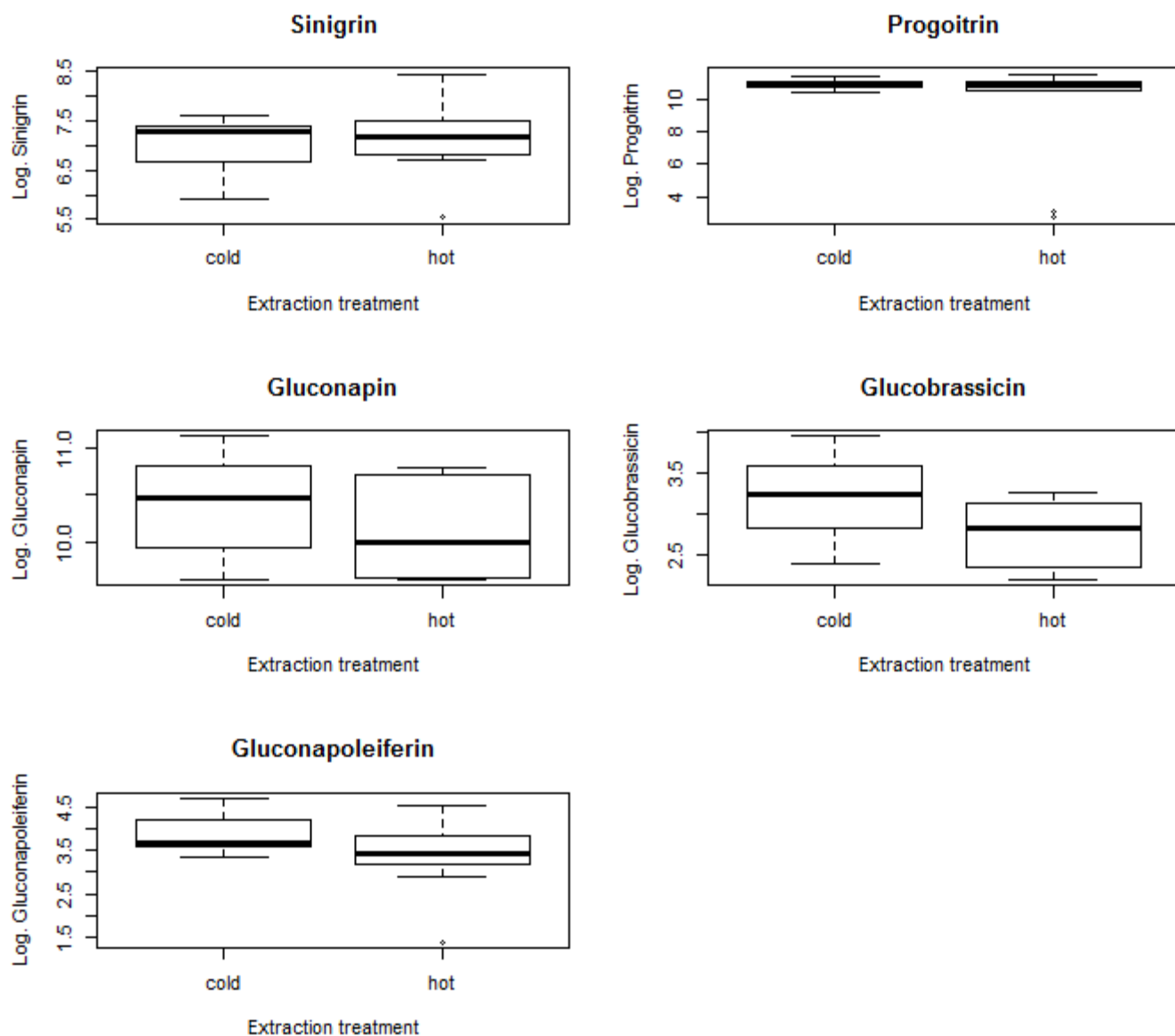


Figure. 9.1. Log transformed concentrations of aliphatic and indole (glucobrassicin) glucosinolates in paired samples of *Brassica oleracea* leaves (n=20) under two different extraction methods, hot 70% MEOH and cold, 80% MEOH. Boxplots show range (whiskers), interquartile range (boxes) and median (thick line).