Bottom-up and top-down herbivore regulation mediated by glucosinolates in

Brassica oleracea* var. *acephala

Author Contributions: SSC, PV, PS, MEC conceived and designed the experiments. SSC, PV, PS, MEC performed the experiments. SSC analyzed the data. SSC wrote the manuscript. PV, PS and MEC contributed to the writing.

Serena Santolamazza-Carbone, Pablo Velasco, Pilar Soengas, María Elena Cartea

Misión Biológica de Galicia, Consejo Superior de Investigaciones Científicas

(MBG-CSIC), P.O. Box 28, 36080, Pontevedra, Spain

Corresponding author: S. Santolamazza-Carbone

Misión Biológica de Galicia (CSIC), P.O. Box 28, 36080, Pontevedra, Spain

E-mail address: anaphes@gmail.com

Short title: Herbivore regulation mediated by glucosinolates in kale crops

Abstract

Quantitative differences in plant defence metabolites, such as glucosinolates, may directly affect herbivore preference and performance, and indirectly affect natural enemy pressure. By assessing insect abundance and leaf damage rate, we studied the responses of insect herbivores to six genotypes of *Brassica oleracea* var. *acephala*, selected from the same cultivar for having high or low foliar content of sinigrin, glucoiberin and glucobrassicin. We also investigated whether the natural parasitism rate was affected by glucosinolates. Finally, we assessed the relative importance of plant chemistry (bottom-up control) and natural enemy performance (top-down control) in

shaping insect abundance, the ratio of generalist/specialist herbivores and levels of leaf damage. We found that high sinigrin content decreased the abundance of the generalist *Mamestra brassicae* (Lepidoptera, Noctuidae) and the specialist *Plutella xylostella* (Lepidoptera, Yponomeutidae), but increased the load of the specialist *Eurydema ornatum* (Hemiptera, Pentatomidae). Plants with high sinigrin content suffered less leaf injuries. The specialist *Brevicoryne brassicae* (Hemiptera, Aphididae) increased in plants with low glucobrassicin content, whereas the specialists *Pieris rapae* (Lepidoptera, Pieridae), *Aleyrodes brassicae* (Hemiptera, Aleyrodidae) and *Phyllotreta cruciferae* (Coleoptera, Chrysomelidae) were not affected by the plant genotype. Parasitism rates of *M. brassicae* larvae and *E. ornatum* eggs were affected by plant genotype. The ratio of generalist/specialist herbivores was positively correlated with parasitism rate. Although both top-down and bottom-up forces were seen to be contributing, the key factor in shaping both herbivore performance and parasitism rate was the glucosinolate concentration, which highlights the impact of bottom-up forces on the trophic cascades in crop habitats.

Key-words: Brassicaceae, herbivory, glucosinolates, parasitoids, tritrophic interactions

Introduction

Plant defence strategies against insect herbivores may involve the synthesis of a plethora of biologically active compounds (allelochemicals) which are phylogenetically conserved in specific plant families or genera (Mithöfer and Boland 2012). Many compounds act directly on the herbivores (bottom-up control), whereas others act indirectly, via the attraction of organisms from other trophic levels (i.e. parasitoids and predators) which, in turn, protect the plants (plant mediated top-down control) (Ode 2006). The genus *Brassica* (Brassicaceae) has a sophisticated two-part defence system involving glucosinolate compounds and a myrosinase protein complex. The enzyme

myrosinase breaks down glucosinolates into toxins (isothiocyanates, nitriles) upon leaf tissue damage (Hopkins et al. 2009). Glucosinolates derived from phenylalanine or tyrosine (aromatic), and those derived from alanine, valine, leucine and isoleucine (aliphatic) are typical of the Brassicales, although they have also been found outside the order, among non-cruciferous dicotyledonous angiosperms (Fahey et al. 2001). Indole (synthesized from tryptophan) and methionine-derived aliphatic glucosinolates are unique to the Brassicales, the latter being a group of metabolites characteristic of the family Brassicaceae (Bekaert et al. 2012). The synthesis of indole glucosinolates, such as glucobrassicin and neoglucobrassicin, tends to be induced by herbivory and fungal infection through jasmonate or other signalling pathways, whereas aliphatic glucosinolates, such as sinigrin and glucoiberin, tend to be constitutively expressed (Harvey et al. 2011). However, it has also been reported that aliphatic compounds may decrease after herbivore attacks (Velasco et al. 2007), and increase after jasmonate induction (Fritz et al. 2010) or by below-ground herbivory (Soler et al. 2005). Thus, patterns and relative concentrations of these chemicals are consistently subject to variation depending on genetic and environmental factors (Poelman et al. 2008; Lankau and Kliebenstein 2009).

Glucosinolates may act as a potent feeding deterrent for generalist insect species, as their toxicity causes developmental and fitness damage. For insects specialized in brassicaceous plants, however, they may act as oviposition and feeding stimulants (kairomonal role). The toxic effect on the specialist herbivores are circumvented by excretion, detoxification, sequestration and behavioural responses (Hopkins et al. 2009). Nonetheless, the existence of qualitative and quantitative variation of phytochemicals among plant genotypes, tissues and ontogenetic stages still challenges insect survival (Ode 2006). Furthermore, glucosinolate breakdown products are also used by the

natural enemies of herbivorous insects, such as parasitoids, as cues for host location. There is evidence that levels of attraction vary between parasitoids, however, and consequently the nature of these secondary metabolites may significantly affect their performance (Turlings and Benrey 1998; Gols and Harvey 2009). The net effect of glucosinolates on the host-parasite interaction is complex. On the one hand, glucosinolates may reduce the immune responses of the hosts, producing a positive effect on parasitoid survival (Bukovinszky et al. 2009), but on the other hand plant metabolites may also negatively affect parasitoid survival, through the direct ingestion of harmful phytochemicals from the herbivore and from reducing host quality (Gols and Harvey 2009). Consequently, plants may face a conflict between producing high or low levels of glucosinolates: higher levels can enhance resistance against generalist insect herbivores, but at the same time may attract co-evolved specialists, and also be harmful to the natural enemies of those herbivores. Lower levels, on the other hand, may increase the abundance of generalist herbivores (Lankau 2007; Kos et al. 2011a).

In *Brassica oleracea* var. *acephala* L. (kale), two aliphatic compounds (sinigrin, glucoiberin) and one indole compound (glucobrassicin) dominate the glucosinolate pattern (Velasco et al. 2007; Cartea et al. 2008). Previous works have focused on the impact of glucosinolates in insect-plant interactions in *Brassica* ecosystems, by studying: 1) different populations of wild *Brassica oleracea* with qualitative differences in glucosinolate patterns (specifically the presence/absence of aliphatic glucosinolates) (Newton et al. 2009a, 2009b; Newton et al. 2010); 2) a wild population of *B. nigra* and cultivated varieties of *B. oleracea* with high or low total glucosinolate concentration (Poelman et al. 2008); 3) different cultivars of *B. oleracea* with quantitative differences in glucosinolate profiles (Poelman et al. 2009); and 4) wild species and cultivated varieties of *B. oleracea*, with quantitative differences in glucosinolate profiles (i.e. high

vs. low levels) (Gols et al. 2008; Harvey et al. 2011). It is known that cultivars of *Brassica* species have low levels of glucosinolates in leaf tissue compared with wild populations, which justifies the comparison between wild and cultivated species (Gols and Harvey 2009). However, it is recognized that other plant traits, such as morphology, phenology, primary chemistry and physiology, related to their different origins, life-histories and genetic backgrounds, could also play a role in insect responses (Carmona et al. 2011). At present, relatively few studies have used artificial selection to create lines of brassicaceous plants with different glucosinolate profiles, and those that did usually only had quantitative variation of sinigrin (Lankau 2007; Lankau and Strauss 2008; Lankau and Kliebenstein 2009). In the present study, we performed a divergent selection from a local variety of kale, obtaining six plant genotypes which shared the phenotype but which differed in glucosinolate patterns, having high or low content of aliphatic (sinigrin and glucoiberin) and indole (glucobrassicin) glucosinolates. We focused on insect responses to quantitative variation in glucosinolates (bottom-up forces), by sampling a wide range of natural occurring specialist and generalist insect herbivores. In particular, generalists were expected to be most sensitive to high sinigrin concentration, as indicated in the literature (Ode 2006). On the other hand, the role of plant chemistry in attracting natural enemies (top-down forces), such as parasitoids, was assessed through the evaluation of parasitism rate. We also determined the extent to which the cost of the synthesis of secondary defence metabolites is translated into benefits (in terms of reduced herbivory), by assessing the variability of leaf damage rates among plant genotypes. Finally, we investigated whether the differences between plant groups in terms of herbivore abundance, the ratio between generalist and specialist herbivores, and leaf damage rates could principally be explained by parasitism rate (top-down forces) or by plant constitutive defences (bottom-up forces).

Material and methods

Plant source

Divergent selection was started in 2006 by using seeds of the kale population MBG-BRS0062 (cycle 0), kept at the *Brassica* germplasm bank at Misión Biológica de Galicia (MBG-CSIC) (Galicia, NW Spain). This population is a local variety which represents the kale germplasm grown in NW Spain. The objective was to obtain six plant groups which had high (H-SIN) or low (L-SIN) concentration of sinigrin, high (H-GIB) or low (L-GIB) glucoiberin, and high (H-GBS) or low (L-GBS) glucobrassicin content. In 2006, approximately 750 plants (cycle 0) were transplanted outside into six cages (125 plants each), and fenced with fine mesh walls to ensure isolation conditions. The leaf glucosinolate content (see details below) of all the plants was assessed 120 days after sowing. In each cage, 20 plants with an extreme content of the relevant glucosinolate (i.e. the highest or the lowest concentration) were selected (20% selection intensity), and all remaining plants were destroyed before flowering. Because kale is an allogamous crop, cross-pollination among the selected plants in each cage was obtained using bumblebees. In 2007, an equal number of seeds were taken from the selected plants of the cycle 0, for each divergent selection, to create the cycle 1 generation (125 plants per cage). According to the protocol adopted for cycle 0, only those plants which showed an extreme leaf glucosinolate content were selected (20 plants per cage). From 2008 to 2009, this process was repeated for two successive generation cycles. A recent investigation, still unpublished, has recorded the absence of significant differences in biomass and phenology between the six plant groups. Thus, we can reasonably conclude that the main differences among genotypes were due to differences in the glucosinolates subjected to selection, although the possibility exists that other plant traits may also combine with the selected glucosinolates to further influence insect performance.

148 *Insect herbivores*

149 The study was focused on insect herbivores feeding on kale leaves. In NW Spain, the
150 most common lepidopteran herbivores are the generalists *Mamestra brassicae* L.
151 (Noctuidae), *Autographa gamma* L. (Noctuidae) and *Evergestis forficalis* L. (Pyralidae),
152 and the specialists *Plutella xylostella* L. (Yponomeutidae), *Pieris rapae* L. (Pieridae)
153 and *P. brassicae* L. (Pieridae) (Cartea et al. 2009). Among hemipterans, the cabbage
154 aphid *Brevicoryne brassicae* L. (Hemiptera, Aphididae), the whitefly *Aleyrodes*
155 *brassicae* Walter (Hemiptera, Aleyrodidae) and the pentatomid *Eurydema ornatum* L.
156 (Hemiptera, Pentatomidae), are also specialist herbivores of brassicaceous plants. The
157 abundance of the adults of the cabbage flea beetle *Phyllotreta cruciferae* Goeze
158 (Coleoptera, Chrysomelidae) was also assessed, because although the larvae feed only
159 on roots and stems, adults feed on the foliage, producing small round holes.

160 *Experimental design*

161 The study was conducted during 2011 and 2012 at Misión Biológica de Galicia. Plants
162 of the six genotypes were grown in multi-pot trays in a greenhouse at 20 °C for 40 days
163 and then transplanted into the field (Salcedo, NW Spain, 42° 24'N, 8° 38'W), at the 5-6
164 true leaf stage, on 15 April 2011 and a second batch on 26 March 2012. Plant varieties
165 were evaluated in a randomized complete block design with six replications. Each
166 experimental block consisted of six rows of 25 plants each (one genotype per row,
167 randomly assigned). Rows were spaced 0.8 m apart and plants within rows were spaced
168 0.5 m apart. Field samplings were performed on 23 May, 23 June and 26 July 2011. A
169 total of 1,080 observations were obtained (corresponding to 60 plants/genotype, three
170 sampling dates and six genotypes). Sampling was interrupted in August 2011 due to the
171 critical conditions of the plants, which were severely affected by whiteflies, aphids and

172 fungal diseases. In 2012, the samplings were carried out on 9 June, 27 July, 27 August,
173 27 September and 30 October. In 2012, 1,800 observations were obtained
174 (corresponding to 60 plants/genotype, five sampling dates and six genotypes). In
175 November, no more insects were found in the field.

176 On each sampling date, ten plants per row were thoroughly inspected in the
177 search for the presence of insect herbivores on leaves. However, the eggs of *A. gamma*,
178 *E. forficalis* and *P. xylostella* and the mining first instar larvae of *P. xylostella*, were not
179 sampled due to their small size. Also, *M. brassicae* pupae were not sampled because
180 they develop while buried in the soil. Plants inspected on one sampling date were
181 always left untouched on the following date. All the lepidopteran species studied are, in
182 Spain, bi- or multivoltine, and we therefore assumed that sampling would not
183 significantly reduce their abundance in the plots. Field collected material (i.e.
184 lepidopteran eggs, larvae and pupae, and *E. ornatum* eggs) was transported to the
185 laboratory, identified, counted and placed in plastic 10cm-diameter-Petri-dishes,
186 labelled with the collection date, host plant, block and insect identity. Larvae were
187 reared individually in Petri-dishes and fed with fresh kale leaves. Lepidopteran
188 immature stages were reared until adulthood or until the emergence of parasitoids. Eggs
189 were maintained until larvae or parasitoids emerged. All rearing was carried out at room
190 temperature (20 ± 2 °C) and under a natural photoperiod. Because of the magnitude of
191 the colonies of *B. brassicae* and *A. brassicae*, and of adults of *E. ornatum* and *P.*
192 *cruciferae*, their abundance was estimated by using a subjective 0-4 rating scale (0 =
193 absence; 1 = up to 5 individuals; 2 = up to 10; 3 = up to 50; 4 = more than 50). It was
194 expressed as the average rating scale measured on 10 plants. The parasitism rate of *B.*
195 *brassicae* and *A. brassicae* was not assessed because these hemipterans are more subject
196 to predators (i.e. syrphid fly maggots, green lacewing larvae, anthocorid bugs and

ladybird beetles) than to parasitoid control in the system studied (S. Santolamazza-Carbone, personal observation). Furthermore, any study of the parasitism rate of these colonies would require several leaves to be removed, thus provoking an alteration of the plant architecture. *E. ornatum* and *P. cruciferae* adults do not suffer parasitoid attacks at this life stage. A 1-5 rating scale was used to evaluate the damage level of the plants (i.e. the overall amount of injuries caused by the whole herbivore complex), where level 1 represents a healthy plant, without any damage, and level 5 represents a completely damaged plant, with 90-100% of the leaves attacked by herbivores. The damage level was calculated as the average rating measured on 15 plants.

The impact of parasitoids on insect host populations was calculated as the proportion of available hosts that had been parasitized per sampling date, block and host plant genotype. Unhatched host eggs, host pupae and unhatched parasitoid cocoons were dissected under the microscope in order to take into account any unemerged adult parasitoids. Parasitoid taxonomical identity was ascertained by S. Santolamazza-Carbone, and voucher specimens have been conserved at Misión Biológica de Galicia.

Glucosinolate analysis

In every experimental plot, two lots of 10 fresh leaves per plant genotype were collected. Collecting dates were 7 June and 7 July 2011, and 10 July and 30 October 2012. Samples were stored at -80 °C until prepared for analysis. Glucosinolate profiles were determined by UHPLC. Sample extraction and desulfation were performed according to Kliebenstein et al. (2001) with minor modifications. 5 microliters of the desulfo-glucosinolate extract from leaves were used to identify and quantify glucosinolates. Chromatographic analyses were carried out on an ultra-high-performance liquid chromatography (UHPLC Nexera LC-30AD; Shimadzu) equipped with a Nexera SIL-30AC injector and one SPD-M20A UV/VIS photodiode array

detector. The UHPLC column was an Acquity UPLC HSS T3 (1.8 μm particle size, 2.1 x100 mm i.d.) from Waters (Waters Corporation, MA, USA) protected with a Van Guard UHPLC precolumn. The oven temperature was set at 30 °C. Compounds were detected at 229 nm and were separated by using the following method in aqueous acetonitrile, with a flow of 0.4 mL min⁻¹: 1.5 minutes at 90% A; a 3.5 min gradient from 10% to 25% (v/v) B; a 4 min gradient from 25% (v/v) to 50% (v/v) B; a 4.5 minute gradient from 50% to 100% (v/v) B; a 1 minute gradient from 100% to 0% (v/v); B and a final 3 min at 90% A. Solvents used were: ultrapure water (A) and 25% of ACN (B). Data were recorded on a computer with the LabSolutions software (Shimadzu). Specific glucosinolates were identified by comparing retention times with standards and by UV absorption spectra. Glucosinolates were quantified at 229 nm by using sinigrin (SIN, sinigrin monohydrate from Phytoflan, Diehm & Neuberger GmbH, Heidelberg, Germany) and glucobrassicin (GBS, glucobrassicin potassium salt monohydrate, from Phytoflan, Diehm & Neuberger GmbH, Heidelberg, Germany) as an external standard and expressed in $\mu\text{mol g}^{-1}$ dry weight (DW). The regression lines were made with at least five data points, from 0.34 to 1.7 nmol for sinigrin and from 0.28 to 1.4 nmol for glucobrassicin. The average regression equations for sinigrin and glucobrassicin were $y = 148818x$ ($R^2 = 0.99$) and $y = 263822x$ ($R^2 = 0.99$), respectively.

Statistical analysis

Sampling dates were analysed as independent events, irrespective of the year, because of the different number of samplings performed in 2011 and 2012. The impacts of plant genotype and sampling date (fixed factors) and the interaction between them on the abundance of insect herbivores and on leaf damage level, were investigated using a two-way analysis of variance (ANOVA). Blocks were used as a random factor. Pairwise comparisons between two plant genotypes (i.e. high vs. low sinigrin, high vs. low

glucoiberin and high vs. low glucobrassicin) and general comparisons (i.e. between the six genotypes), were also carried out. Insect count data were $\log_{10}(x+1)$ transformed prior to the analyses, whereas the insect abundance estimated by rating scales and the leaf damage level were arc-sin-square root transformed. Differences between means were assessed by a LSD (Least Significant Difference) test. The larvae of *P. brassicae*, *E. forficalis* and *A. gamma*, and the eggs of *P. rapae* were excluded from the statistical analyses because they were only sporadically found.

Differences in the ratio of generalist (i.e. *M. brassicae*, *A. gamma*, *E. forficalis*) to specialist (i.e. *P. xylostella*, *P. rapae*, *P. brassicae*, *E. ornatum*) herbivores among plant genotypes and sampling dates (fixed factors) were assessed by using a Generalized Linear Model with binomial proportion (logistic regression) and logit link function.

The influence of plant genotype, sampling date and the interactions between them on the parasitism rate was assessed by means of Generalized Linear Model (logistic regression) with binomial proportion and logit link function. The binomial proportion (i.e. number of parasitized hosts/number of available hosts) was treated as the response variable, whereas plant genotype and sampling date were the independent variables. Pairwise and general comparisons among genotypes were assessed. Parasitism rate was only assessed for immature stages of the lepidopteran species and *E. ornatum* eggs.

In order to assess whether overall herbivore abundance, the ratio of generalist to specialist herbivores and the leaf damage level were correlated with glucosinolate concentrations or with the parasitism rate (fixed factors), a multiple linear regression was adopted. Glucosinolate concentrations were expressed as the mean values for each sample date (June and July in 2011, and July and October in 2012), with six replications per genotype. For each glucosinolate, data from plants selected for having high and low

concentrations were pooled. Parasitism rate was arc-sin square root transformed and herbivore abundance was $\log_{10}(x+1)$ transformed prior to the analysis. Significance was declared at $P < 0.05$. Statistical tests were carried out by using the GenStat12.1 software package (VSN International Ltd, Hemel Hempstead, UK).

Results

Glucosinolates

Glucosinolate analyses confirmed that in both years the mean concentrations of sinigrin, glucoiberin and glucobrassicin varied significantly between genotypes (high vs. low concentration), according to the divergent selection previously performed (online resource, Table S1, ESM). In some cases, significant variation between genotypes in the concentration of other glucosinolates, not subjected to divergent selection, was also detected. In fact, this outcome was to be expected owing to the existence of links between the biosynthetic pathways of the different glucosinolates (Fahey et al. 2001). However, this factor is unlikely to have affected the reliability of the study because these variations were of considerably lower magnitude than those recorded in the selected glucosinolates.

Herbivore responses to glucosinolate patterns

Variation in the herbivore numbers recorded between the two years was due to natural population fluctuations and to the different number of sampling events (Table 1). We found that *M. brassicae* (84%) dominated the lepidopteran community, followed by *P. rapae* (8%) and *P. xylostella* (5%). Pairwise comparison of plant genotypes showed that the leaf damage level was significantly lower in plants with high sinigrin content (Table 2, Fig. 1-a). General comparisons across the six plant genotypes also showed the high sinigrin genotype to have significantly lower leaf damage, as well as a significant

variation in the extent of leaf damage depending on the sampling date (Table 3, Fig. 1-a).

Among the lepidopterans, *M. brassicae* larvae were significantly less abundant on the high sinigrin content genotype (pairwise comparisons) (Table 2, Fig. 1-b). By performing general comparisons, we found that plant genotype, sampling date and the interactions between them exerted a significant effect on the larval stage (Table 3), which avoided the plants selected for having high glucoiberin content (Fig. 1-b). The abundance of *M. brassicae* eggs significantly decreased on the plants with high sinigrin concentration, but also on the genotype with low glucoiberin content (pairwise comparisons) (Table 2, Fig. 1-c). However, general comparisons only detected significant effects of the sampling date (Table 3).

Pairwise comparisons between genotypes showed *P. xylostella* larvae and pupae to be less abundant in the plant genotype with high sinigrin content (Table 2, Fig. 1-d); however no genotype effect was observed on *P. rapae* larvae or pupae abundance (Table 2), although populations of this species did fluctuate depending on sampling date (Fig. 1-e). When general comparisons were performed, only the sampling date was significant for both *P. xylostella* and *P. rapae* (Table 3).

Among the hemipterans, the abundance of *E. ornatum* eggs significantly increased in plants with high sinigrin content, and also varied depending on the sampling date (pairwise comparisons, Table 2, Fig. 1-f). General comparisons confirmed the attraction exerted by plants with high sinigrin concentration (Table 3, Fig. 1-f). Numbers of adult *E. ornatum*, however, were only significantly affected by the sampling date, as indicated by both pairwise (Table 2, Fig. 1-g) and general comparisons (Table 3).

The size of the colonies of *B. brassicae* consistently increased in plants with low glucobrassicin concentration and depended on the sampling date (pairwise comparisons) (Table 2, Fig. 1-h). When considering general comparisons, only the sampling date and the interaction between sampling date and plant genotype were found to be significant for this species (Table 3).

The colonies of the whitefly *A. brassicae* (Fig. 1-i) and adults of the coleopteran *P. cruciferae* (Fig. 1-l) varied their magnitude depending on the sampling date in both pairwise (Table 3) and general comparisons (Table 3).

The ratio of generalist to specialist herbivores significantly differed among plant genotypes (Wald test = 80, $P < 0.001$) and sampling dates (Wald test = 431.7 $P < 0.001$; genotype $\times$ sampling date: Wald test = 313.54, $P < 0.001$). In particular, when both years are considered together, plants selected for having high sinigrin and low glucobrassicin content were seen to be significantly more visited by specialists than generalists (online resource, Fig. S1-a, ESM). Considering the two years separately, specialists dominated all plant groups in 2011, but preferred the genotype with high sinigrin concentration (online resource, Fig. S1-b, ESM); in 2012, however, generalists were more abundant, especially in plants with low sinigrin, and high or low glucobrassicin content (online resource, Fig. S1-c, ESM).

Parasitoid responses to glucosinolate patterns

Lepidopterans were parasitized by hymenopteran parasitoids belonging to the Braconidae, Ichneumonidae, Encyrtidae, Pteromalidae, Eulophidae, Scelionidae and Trichogrammatidae families, and by tachinid dipterans, whereas the eggs of the hemipteran *E. ornatum* were attacked by *Trissolcus* sp. (Hymenoptera, Scelionidae) (see Santolamazza-Carbone et al. 2013 for details of the parasitoid complex of *B. oleracea* herbivores). In 2011, the parasitism rate experienced by the generalist herbivores was

lower (0.12 ± 0.01) than that experienced by the specialists (0.26 ± 0.03). A similar trend was found in 2012 for generalists (0.15 ± 0.05) and specialists (0.41 ± 0.01).

The parasitism of *M. brassicae* larvae (Table 1), mainly exerted by the braconid wasps *Microplitis mediator* L. and, occasionally, by *Cotesia rubecula* Marshall, was significantly greater in those plants selected for high glucoiberin and high glucobrassicin content (pairwise comparisons) (Table 4). When considering general comparisons (Table 5), it was found that the parasitism rate was higher in genotypes with high glucoiberin and high glucobrassicin content (Table 1), and also affected by sampling date, although the interaction was not significant

When performing pairwise comparisons, we found that plant genotypes did not influence the parasitism rate of *M. brassicae* eggs, or of *P. rapae* and *P. xylostella* larvae (Table 1 and 4). Similar results were obtained from general comparisons of plant genotypes for the parasitism rate of *M. brassicae* eggs (Table 5). Sampling date did significantly affect the parasitism rate of *P. rapae* larvae (general comparisons), but the interaction between genotype and sampling date did not (Table 5). The parasitism rate of *P. xylostella* larvae was not affected by the plant genotype, the sampling date or their interactions when considering both pairwise (Table 4) and general comparisons (Table 5).

In 2011, 13.5% of *E. ornatum* eggs were parasitized, and parasitism rate was highest in plants with low sinigrin and high glucobrassicin content (Table 1 and 4). In 2012 we did not detect any parasitism (Table 1). Further analysis (general comparisons) confirmed the positive effect of high glucobrassicin plants on the egg parasitism rate of this hemipteran, as well as the importance of the sampling date (Table 5).

Top-down vs. bottom-up effects

In 2011, herbivore abundance was significantly and positively correlated with sinigrin and glucobrassicin content, while the parasitism rate calculated in these plant genotypes was not (Table 6). The leaf damage rate was significantly and negatively correlated with sinigrin concentration, while the parasitism rate did not produce any effect on it (Table 6). The ratio of generalist to specialist herbivores calculated on the genotypes with high and low glucobrassicin content did not show any significant relationship with glucosinolate concentration, but it had a positive relationship with the parasitism rate (Table 6).

In 2012, variation in sinigrin, glucoiberin and glucobrassicin concentrations or parasitism rate did not have any significant relationship with herbivore abundance (Table 6). However, sinigrin concentration and the parasitism rate were significantly and negatively related to the leaf damage rate (Table 6). The ratio between generalist and specialist herbivores was not related to glucosinolate concentration or to parasitism rate (Table 6).

Discussion

The study indicates that quantitative variations in glucosinolate profiles, and in particular variations in sinigrin content, influence both the overall abundance of insect herbivores and the relative proportion of specialist and generalist species, which is translated into a significant variation in leaf damage levels. Sinigrin is known to be the principal glucosinolate found in kale varieties of NW Spain (Cartea et al. 2008), and this investigation shows that a high concentration of this major chemical defence can help deter both generalist and specialist lepidopteran herbivores. However, high sinigrin content may allow for an increased load of specialist hemiptera, and this can lead to an overall positive correlation between sinigrin concentration and herbivore abundance.

This means that the role of specialist hemiptera on altering the net value of plant defence traits could be crucial, as also reported by Lankau (2007). Furthermore, the third trophic level represented by hymenopteran and dipteran parasitoids also performed differently depending on the plant genotype, which is likely to be due to variation in both the host density and the volatile blends emitted by the host plants (Gols and Harvey 2009).

Herbivore responses to glucosinolate patterns

Host plants with high sinigrin and low glucobrassicin concentrations harboured a significantly higher load of specialists. In particular, lepidopterans were outnumbered by specialist hemipterans in 2011, especially in plant groups with high sinigrin content, although this did not occur in 2012. There are a number of reasons that may explain the wide distribution of the hemipterans across the plant genotypes: for example, insects may build up high numbers on preferred host plants, eventually spilling over onto less preferred ones (associational susceptibility) (White and Whitam 2000). Furthermore, variation in the ratio between generalists and specialists also depended on the temporal components of herbivory: generalist species tended to avoid plants previously consumed by specialists, whereas specialists often colonized in large numbers plants that were already being consumed by other phytofagous insects, irrespective of their feeding mode (Poelman et al. 2010).

As expected, egg and larval abundances of *M. brassicae* were lower in plants with high sinigrin concentration. Attraction of the *M. brassicae* female to patches of plants may be mediated by visual and biochemical cues, while the decision of where to oviposit within a plant population would depend on the specific plant chemistry. The response of *M. brassicae* larvae to glucoiberin is less obvious. On the one hand, larvae were less abundant in September in plants with a high content of this aliphatic

glucosinolate; this agrees with previous studies showing high glucoiberin concentrations to be negatively correlated with herbivore abundance and species richness (Poelman et al. 2009; Kos et al. 2011a). However, it was also found that *M. brassicae* eggs were more abundant in plants with high glucoiberin concentration. These discrepancies are commonly found in the literature, which reports impacts of the host plant on the *M. brassicae* fitness components ranging from no effect (Newton et al. 2009), to effects similar to those experienced by specialists herbivores (Poelman et al. 2008), to negative effects on larval survival, especially those genotypes with high concentration of aliphatic glucosinolates (Gols et al. 2008; Harvey and Gols 2011).

In agreement with previous studies on neutral *P. rapae* responses to change in glucosinolate patterns (Newton et al. 2009; Newton et al. 2010; Gols et al. 2008), but in contrast with others where the negative effect of indole glucosinolates on oviposition preference has been reported (de Vos et al. 2008), we found that *P. rapae* did not respond to the range of glucosinolate profiles. It has been reported that this butterfly can redirect the course of the normal hydrolysis reaction that is provoked by the enzyme myrosinase upon insect feeding, by producing nitriles instead of toxic isothiocyanates, which are then excreted by the larvae (Hopkins et al. 2009). This detoxification system may allow *P. rapae* to be relatively insensitive to changes of plant chemical concentrations, as reported by Harvey et al. (2007).

Several studies on host plant recognition have reported the ability of *P. xylostella* to employ glucosinolates as olfactory cues for oviposition (Hopkins et al. 2009). In particular, the role of aliphatic glucosinolate breakdown products (Spencer et al. 1999; Renwick et al. 2006) and the impact of intact indole glucosinolates on oviposition behaviour (Reed et al. 1989; Sun et al. 2009) have been shown. Furthermore, as a crucifer specialist, physiological counter-adaptations to plant defence

compounds have been evolved by *P. xylostella* larvae, which possess a glucosinolate sulfatase enzyme in the gut that enables the conversion of glucosinolates to desulfoglucosinolates, rather than toxic nitriles and isothiocyanates (Raztka et al. 2002). Interestingly, we found that *P. xylostella* abundance did not increase in plants with high sinigrin content, which does not agree with the evidence that, under laboratory conditions, elevated sinigrin concentration is highly attractive to this moth (Spencer et al. 1999).

Piercing-sucking insects, such as hemipterans, are exposed to intact glucosinolate or possibly to the by-product produced by damages provoked by other herbivores (Hopkins et al. 2009). The aphid *B. brassicae* is a glucosinolate-sequestering specialist herbivore, which uses aphid-specific myrosinase enzymes to form toxic hydrolytic products against its natural enemies (Cole 1997). Among the hemipteran species studied, only *B. brassicae* showed significant responses to glucobrassicin variation, being more abundant on the low glucobrassicin genotype. This finding is consistent with the evidence that the sequestration of glucosinolates by this aphid from plant phloem is selective, with a clear preference for aliphatic instead of indole secondary metabolites (Kos et al. 2011b). It is interesting that this herbivore, which has a passive dispersal mechanism, displayed a clear response to certain glucosinolate profiles.

This is the first time that the performance of the pentatomid *E. ornatum* in response to glucosinolate polymorphism of the host plant has been investigated. Although the adult bugs did not show preference for a specific plant genotype, their egg masses were especially abundant in plants with a high sinigrin content. The role of sinigrin as an oviposition stimulant for the *E. ornatum* female has never been reported in the past. A previous study on the responses of this hemipteran to the glucosinolate

pattern of different *Brassica* crops did, however, highlight the importance of having a high content of aliphatic compounds, such as progoitrin, epiprogoitrin, gluconapin and glucoraphanin, and of the indole glucobrassicin in order to reduce the extent of damage, although the effects also depended on plant age (Bohinc et al. 2013).

Parasitoid responses to glucosinolate patterns

Parasitization of insect hosts is the result of a complex process which involves the attraction of parasitoids by semiochemical cues emitted by both the host (kairomones) and the plant (synomones), parasitoid arrestment and host searching, host selection, acceptance and oviposition (Godfray 1994). In the present study, the proportion of herbivores that were parasitized was affected by differences in the herbivore-induced volatile emissions of the different plant genotypes. Herbivore density was not manipulated because we were also interested in measuring herbivore abundance on different plant genotypes, and investigating its impact on the parasitism rate. However, the host density-dependent effect was not seen to contribute to top-down control. *E. ornatum* eggs, for example, were more abundant in plants with high sinigrin content, but suffered from a higher parasitism rate in plants with low sinigrin content. *M. brassicae* larvae were more abundant in plants with low glucoiberin content but not more parasitized there. Similarly, *M. brassicae* larvae and *E. ornatum* eggs were more parasitized in plants with high glucobrassicin content, even though their populations were not especially high on this genotype. These findings agree with work carried out on other *B. oleracea* varieties, which has shown volatile indole-derivates to be important in the attraction of natural enemies of pentatomid bugs (Conti et al. 2008). In fact, egg oviposition by herbivores could induce specific plant responses which are relevant for host location by egg parasitoids (Fatouros et al. 2012).

Top-down vs. bottom-up effects

Whether or not plant resources, natural enemies, or both, determine the abundance of insect herbivores in natural multi-trophic systems has long been a topic of debate (Hunter and Price 1992; Halaj and Wise 2001) that is yet to reach a general consensus. In comparison with natural habitats, managed crop systems have a relatively simple food web structure, characterized by the presence of a homogeneous plant community, for which the impact of bottom-up and top-down forces appears more predictable. The higher propensity of crop habitats to experience strong trophic cascades was clearly shown through meta-analysis by Halaj and Wise (2001). In this type of habitat the reduction of predator abundance, more than plant chemical defences, generally increased herbivory and reduced primary plant production (Halaj and Wise 2001). Our results, however, indicate the opposite trend. In our study system, the impact of bottom-up forces on insects agrees with the general view about the importance of plant mediated forces in terrestrial ecosystems (Denno et al. 2002). Furthermore, previous studies focused on brassicaceous plants (Newton et al. 2009b; Kos et al. 2011a) have also highlighted that plant chemistry and morphology have more impact than natural enemy activity in shaping herbivore abundance.

The statistical analyses showed that glucosinolate concentrations have a significant effect on the overall herbivore abundance, on the relative abundance of generalist and specialist herbivores, on the parasitism rate and on the leaf damage rate. On the other hand, parasitism rate also contributed in shaping the leaf damage rate in 2011, and the ratio between generalist and specialist insects in 2012, which suggests that in reality many ecological forces combine to determine the patterns observed in the field. Interestingly, in 2011 the increase in sinigrin content was positively correlated with herbivore abundance, whereas an opposite trend was found for leaf damage rate. The massive presence of the hemipteran *E. ornatum*, and in particular the preference

manifested by ovipositing females for plants with high sinigrin content, explained the positive relationship between sinigrin concentration and herbivore abundance. The reduction in leaf damage, on the other hand, can be explained by the fact that although overall herbivore numbers increased with high sinigrin content, the numbers of generalist lepidopteran herbivores decreased. This prompted a net decline in overall leaf damage because the damage provoked by chewing insects (including Lepidoptera) was disproportionately high and more widely detected than damage from hemipteran sap-sucking species. In 2012, the leaf damage rate in plants with high sinigrin content was also reduced as a result of the increase in parasitism rate among the lepidoptera (the same pattern was not seen in hemiptera: *E. ornatum* eggs, for example, were parasitized in 2011 but not in 2012). These findings highlighted the role of sinigrin in plant protection. However, an ecological cost of plant defence through sinigrin synthesis does exist, because specialists were more attracted to a high concentration of this secondary metabolite. Previous field studies on the role of *B. oleracea* chemicals on insect herbivore biodiversity showed the impact of glucoiberin in shaping insect communities (Poelman et al. 2009; Kos et al. 2011a). Data supporting the idea that sinigrin influences herbivore choices comes from laboratory trials (Shields and Mitchell 1995; Gols et al. 2008) and from field tests performed in controlled environments (by manipulating insect presence and abundance) (Lankau 2007; Lankau and Kliebenstein 2008; Lankau and Strauss 2009; Kos et al. 2011a). This is the first time that an investigation under natural conditions reported significant responses to sinigrin, glucoiberin and glucobrassicin expressed by herbivores with different feeding modes and behavioural ecologies, and by their parasitoids.

To conclude, our results illustrate how quantitative variation in aliphatic and indole glucosinolates of kales may influence herbivore abundance and the control

exerted by the parasitoid complex. Furthermore, the plant glucosinolate pattern contributes to reducing leaf damage rate. Although the top-down force indeed acts in concert with bottom-up regulation and with the other environmental factors, parasitoid pressure seems to be a weaker force in our study system.

Acknowledgements

Research was supported by the National Plan for Research and Development (AGL2012-35539). Authors thank Rosaura Abilleira, Pilar Comesaña, César González, and Tamara Sotelo for their valuable help in field samplings. We also thank David Brown for improving the English. S. Santolamazza-Carbone acknowledges a post-doc research contract (JAE-Doc) from the CSIC.

References

- Bekaert M, Edger PP, Hudson CM, Pires JC, Conant GC (2012) Metabolic and evolutionary costs of herbivory defense: systems biology of glucosinolate synthesis. *New Phytologist* 196: 596-605
- Bohinc T, Hrastar R, Kosir IJ, Trdan S (2013) Association between glucosinolate concentration and injuries caused by cabbage stink bugs *Eurydema* spp. (Heteroptera: Pentatomidae) on different Brassicas. *Acta Scientiarum. Agronomy* 35:1-8
- Bukovinszky, T, Poelman EH, Gols R, Prekatsakis G, Vet LEM, Harvey JA, Dicke M (2009) Consequences of constitutive and induced variation in plant nutritional quality for immune defence of a herbivore against parasitism. *Oecologia* 160: 299-308
- Carmona D, Lajeunesse M J, Johnson MTJ (2011) Plant traits that predict resistance to herbivores. *Funct Ecol* 25:358-367

569 Cartea M E, Padilla G, Vilar M, Velasco P (2009) Incidence of the major *Brassica* pests
570 in northwestern Spain. J Econ Entomol 102:767-773

571 Cartea ME, Velasco P, Obregón S, Padilla G, de Haro A (2008) Seasonal variation in
572 glucosinolate content in *Brassica oleracea* crops grown in northwestern Spain.
573 Phytochemistry 69:403-410

574 Cole RA (1997) The relative importance of glucosinolates and amino acids to the
575 development of two aphid pests *Brevicoryne brassicae* and *Myzus persicae* on
576 wild and cultivated brassica species. Entomol Exp Appl 85:121-133

577 Conti E, Zandra C, Salerno G, Leonbruni B, Volpe D, Frati F, M C, Bin F (2008)
578 Changes in the volatile profile of *Brassica oleracea* due to feeding and
579 oviposition by *Murgantia histrionica* (Heteroptera: Pentatomidae). Europ J
580 Entomol 105:839-847

581 Denno RF, Gratton C, Peterson MA, Langellotto GA, Finke DL, Huberty AF (2002)
582 Bottom-up forces mediate natural-enemy impact in a phytofagous insect
583 community Ecology 83:1443–1458

584 de Vos M, Kriksunov KL, Jander G (2008) Indole-3-acetonitrile production from indole
585 glucosinolates deters oviposition by *Pieris rapae* (white cabbage butterfly).
586 Plant Physiol 146: 916-926

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

589 Fatouros NE, Lucas-Barbosa D, Weldegergis BT, Pashalidou FG, van Loon JJA, Dicke
590 M, Harvey JA, Gols R, Huigens ME (2012) Plant volatiles induced by herbivore
591 egg deposition affect insects of different trophic levels. PLoS ONE 7(8):
592 e43607. doi:10.1371/journal.pone.0043607

593 Fritz VA, Justen VL, Bode AM, Schuster T, Wang M (2010) Glucosinolate
594 enhancement in cabbage induced by jasmonic acid application. HortScience 45:
595 1188-1191

596 Godfray HCJ (1994) Parasitoids. Behavioral and Evolutionary Ecology. Princeton
597 University Press, Princeton

598 Gols R, Bukovinszky T, van Dam N, Dicke M, Bullock JM, Harvey JA (2008)
599 Performance of generalist and specialist herbivores and their endoparasitoids
600 differs on cultivated and wild *Brassica* populations. J Chem Ecol 34:132-143

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

603 Halaj J, Wise DH (2001) Terrestrial trophic cascades: how much do they trickle?
604 American Naturalist 157: 262-281

605 Harvey JA, Gols R (2011) Population-related variation in plant defense more strongly
606 affects survival of an herbivore than its solitary parasitoid wasp. J Chem Ecol
607 37:1081-1090

608 Harvey JA, Gols R, Wagenaar R, Bezemer TM (2007) Development of an insect
609 herbivore and its pupal parasitoid reflect differences in direct plant defense. J
610 Chem Ecol 33:1556-1569

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

614 Hopkins RJ, van Dam N, van Loon JJA (2009) Role of glucosinolates in insect-plant
615 relationships and multitrophic interactions. Ann Rev Entomol 54:57-83

616 Hunter MD, Price PW (1992) Playing chutes and ladders: heterogeneity and the relative
 617 role of bottom-up and top-down forces in natural communities. *Ecology* 73:
 618 724-732

619 Kliebenstein D, Lambrix V, Reichelt M, Mitchell-Olds T (2001) Gene duplication and
 620 the diversification of secondary metabolism: side chain modification of
 621 glucosinolates in *Arabidopsis thaliana*. *Plant Cell* 13: 681-693

622 Kos M, Broekgaarden C, Kabouw P, Oude Lenferink K, Poelman EH, Vet LEM., Dicke
 623 M, van Loon JJA (2011a) Relative importance of plant-mediated bottom-up and
 624 top-down forces on herbivore abundance on *Brassica oleracea*. *Funct Ecol*
 625 25:1113-1124

626 Kos M, Kabouw P, Noordam R, Hendriks K, Vet LEM, van Loon JJA, Dicke M
 627 (2011b) Prey-mediated effects of glucosinolates on aphid predators. *Ecol*
 628 *Entomol* 36: 377-388

629 Lankau RA (2007) Specialist and generalist herbivores exert opposing selection on a
 630 chemical defense. *New Phytol* 175:176-184

631 Lankau RA, Strauss SY (2008) Community complexity drives patterns of natural
 632 selection on a chemical defence of *Brassica nigra*. *Am Nat* 171: 150-161

633 Lankau RA, Kliebenstein DJ (2009) Competition, herbivory and genetics interact to
 634 determine the accumulation and fitness consequences of a defence metabolite. *J*
 635 *Ecol* 97:78-88

636 Mithöfer A, Boland W (2012) Plant defense against herbivores: chemical aspects. *Ann*
 637 *Rev Plant Biol* 63:431-450

638 Newton EL, Bullock JM, Hodgson DJ (2009a) Bottom-up effect of glucosinolate
 639 variation on aphid colony dynamics in wild cabbage populations. *Ecol Entomol*
 640 34:614-623

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

644 Newton EL, Bullock JM, Hodgson DJ (2010). Temporal consistency in herbivore
 645 responses to glucosinolate polymorphism in populations of wild cabbage
 646 (*Brassica oleracea*). *Oecologia* 164:689-699

647 Ode P (2006) Plant chemistry and natural enemy fitness: effect on herbivore and natural
 648 enemy interactions. *Ann Rev Entomol* 51:163-185

649 Poelman EH, Broekgaarden C, van Loon JJA, Dicke M (2008) Early season herbivore
 650 differentially affects plant defence responses to subsequently colonizing
 651 herbivores and their abundance in the field. *Mol Ecol* 17:3352-3365

652 Poelman EH, van Dam N, van Loon JJA, Vet LEM, Dicke M (2009) Chemical diversity
 653 in *Brassica oleracea* affects biodiversity of insect herbivores. *Ecology* 90:1863-
 654 1877

655 Poelman EH, van Loon JJA, van Dam N, Vet LEM, Dicke M (2010) Herbivore-induced
 656 plant responses in *Brassica oleracea* prevail over effects of constitutive
 657 resistance and result in enhanced herbivore attack. *Ecol Entomol* 35:240-247

658 Raztka A, Vogel H, Kliebenstein DJ, Mitchell-Olds T, Kroymann J (2002) Disarming
 659 the mustard oil bomb. *Proc Nat Acad Sci, USA* 99:11223-11228

660 Reed DW, Pivnick KA, Underhill EW (1989) Identification of chemical oviposition
 661 stimulants for the diamondback moth, *Plutella xylostella*, present in three
 662 species of Brassicaceae. *Entomol Exp Appl* 53, 277-286

663 Renwick JA, Haribel M, Gouinguéné S, Städler E (2006) Isothiocyanates stimulating
 664 oviposition by the diamondback moth, *Plutella xylostella*. *J Chem Ecol* 32: 755-
 665 766

666 Santolamazza-Carbone S, Velasco P, Selfa J, Soengas P, Cartea ME (2013)
 667 Intraspecific variation of host plant and locality influence the lepidopteran-
 668 parasitoid system of *Brassica oleracea* crops. J Econ Entomol 106: 1134-1144
 669 Spencer JL, Pillai S, Bernays EA (1999) Synergism in the oviposition behavior of
 670 *Plutella xylostella*: sinigrin and wax compounds. J Insect Behav 12:483-500
 671 Soler R, Martijn Bezemer T, Van der Putten WH, Vet LEM, Harvey JA (2005) Root
 672 herbivore effects on above-ground herbivore, parasitoid and hyperparasitoid
 673 performance via changes in plant quality. J Anim Ecol 74: 1121-1130
 674 Sun JY, Sonderby IE, Halkier BA, Jander G, de Vos M (2009) Non-volatile intact
 675 indole glucosinolates are host recognition cues for ovipositing *Plutella*
 676 *xylostella*. J Chem Ecol 35: 1427-1436
 677 Turlings TCJ, Benrey B (1998) Effects of plant metabolites on the behavior and
 678 development of parasitic wasps. Écoscience 5:321-333
 679 Velasco P, Cartea ME, González C, Vilar M, Ordás A (2007) Factors affecting the
 680 glucosinolate content of kale (*Brassica oleracea acephala* group). J Agr Food
 681 Chem 55: 955-962
 682 White JA, Whitham TG 2000 Associational susceptibility of cottonwood to a box elder
 683 herbivore. Ecology 81: 1795-180